Isolation and characterisation of phytotoxins produced by the *Botryosphaeriaceae* and their role in grapevine trunk diseases

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Certificate of Authorship

“I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

I agree that this thesis be accessible for the purpose of study and research in accordance with normal conditions established by the Executive Director, Library Services, Charles Sturt University or nominee, for the care, loan and reproduction of thesis, subject to confidentiality provisions as approved by the University.”

Signed:

Pierluigi Reveglia Date: 7 August 2019
Publications and Manuscripts Arising from the Research

Refereed Journal Articles:

Paper 1


Paper 2


Paper 3


Paper 4


Paper 5

**Author Contribution Statements**

**Paper 1.** PR, SS, RBB, MM and AE selected the fungal isolate and assisted in the experimental design. PR (candidate) performed the experiment, interpreted the data and wrote the manuscript. RBB and SS supervised the culturing of fungi and the bioassay on grapevine leaves. MM and AE provided advice on the chromatographic purification and NMR data analysis. SS, RBB, MM assisted in editing the manuscript.

**Paper 2.** PR, SS, RBB, AC and AE selected the fungal isolate and assisted in the experimental design. PR (candidate) performed the experiment, interpreted the data and wrote the manuscript. RBB and SS supervised the culturing of fungi and the bioassay on grapevine leaves. AC and AE provided advice on the chromatographic purification and NMR data analysis. SS, RBB, AC and AE assisted in editing the manuscript.

**Paper 3.** PR, SS, RBB, MM, AC and AE selected the fungal isolates and assisted in the experimental design. PR (candidate) performed the experiment, interpreted the data and wrote the manuscript. RBB and SS supervised the culturing of fungi and the bioassay on grapevine leaves. MM, AC and AE provided advice on the chromatographic purification and NMR data analysis. SS, RBB, MM, AC and AE assisted in editing the manuscript.

**Paper 4.** PR, SS, RBB and AE selected the fungal isolate and assisted in the experimental design. PR (candidate) performed the experiment, interpreted the data and wrote the manuscript. RBB and SS supervised the culturing of fungi and the bioassay on grapevine leaves. MM, AC and AE provided advice on the chromatographic purification and NMR data analysis. SS, RBB, MM, AC and AE assisted in editing the manuscript.

**Paper 5.** PR, SS, RBB set up the experiment. PR (candidate) performed the experiment, optimised the method, interpreted the data and wrote the manuscript. RBB and SS supervised the molecular biology analysis. MM, AC and AE assisted with the mass spectrometry data analysis. SS, RBB assisted in editing the manuscript.
"I declare that the author contribution statements above are accurate, and I give permission for the candidate to include the papers and manuscripts in this thesis."

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I began this experience as an enthusiastic young chemist who had just finished his Master degree; now, three years later, I am aware that only a multidisciplinary approach based on environmentally friendly choices would help humankind to solve the scientific challenges of our century.

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“For every minute spent in organizing, an hour is earned.”

— Benjamin Franklin

“Above all, don’t fear the difficult moments. The best comes from them.”

— Rita Levi Montalcini

“With a great power comes great responsibility”

— Peter Benjamin Parker
Abstract

Grapevine trunk diseases (GTDs) represent a serious threat to vineyards worldwide causing substantial economic and yield losses. To date, no curative methods are available and the connection between pathogen and symptom expression is not completely understood. Among the GTDs, Botryosphaeria dieback (BD) is considered a serious problem worldwide causing cankers, dieback and eventually death of vines. At least 27 species of the Botryosphaeriaceae are associated with BD worldwide, although only 11 species have been reported in Australian vineyards to date. In Europe, the disease is also associated with foliar symptoms. However, these symptoms have not been reported in Australian vineyards to date. The expression of foliar symptoms is usually associated with phytotoxic metabolites (PMs) produced by the pathogen that are translocated into the leaves. Prior to this investigation, limited information was available on the PMs produced by Australian isolates of Botryosphaeriaceae spp.

The most widespread and most virulent species of Botryosphaeriaceae involved in BD in South Australia (SA) and New South Wales (NSW) vineyards were selected. The chromatographic profiles of organic extracts from culture filtrates of eight species of Botryosphaeriaceae showed the ability of all isolates to produce several and different metabolites. The phytotoxicity of the organic extracts varied among isolates and species when tested on grapevine leaves and tomato cuttings. The PMs produced by Diplodia seriata H141a, Dothiorella mutila DAR79137, Neofusicoccum austral e DAR79506, N. parvum B19, and for the first time, those produced by N. luteum DAR81016, Spencermartinsia viticola DAR78870 and Do. vidmadera DAR78993 were isolated and fully characterised by spectroscopic method (1D and 2D NMR and HR ESIMS). Furthermore, the exopolysaccharides (EPSs) produced by three Neofusicoccum spp. were isolated and assayed on grapevine leaves in vitro and all the EPSs were shown to be phytotoxic at different concentrations.

The in planta production of PMs was investigated by molecular and analytical chemistry techniques. Wood samples from vines naturally-infected with BD were collected from three vineyards in NSW. In addition, one-year-old rootlings (cvs. Chardonnay and Cabernet Sauvignon) were inoculated with D. seriata H141, S. viticola DAR78870 and Do. vidmadera DAR78993. All symptomatic and asymptomatic wood samples were analysed by cultural isolations, quantitative PCR (qPCR) and LC-QqQ in MRM mode. (R)-mellein was only detected in symptomatic wood samples and its amount was correlated with the amount of pathogen DNA detected by qPCR. These results suggest that (R)-
mellein was not translocated throughout the wood as previously hypothesised, and foliar symptoms may be caused by a combination of diverse factors which require further in-depth studies.

Genetic analysis of the pathogen genome was also attempted to identify pathogenicity and virulence genes involved in the production of (R)-mellein. The production of (R)-mellein by _D. seriata_ A141a and _N. parvum_ DAR78998 was confirmed by HPLC quantification. Several sets of primers previously developed for the amplification of the polyketide synthase (PKS) gene of _Parastagonospora nodorum_ were optimised and tested on isolates of _D. seriata_ and _N. parvum_. Only one set of primers was suitable for _N. parvum_. The BLASTn analysis for resulting sequences showed the highest similarity with the putative gene involved in the expression of the 6-methyl-salicylic acid (6-MSA) synthase protein in _D. corticola_ and _N. parvum_. This protein is a PKS and it is likely that the amplified sequence encodes for the biosynthesis of a partially reduced polyketide (PK) compound similar to 6-MSA such as (R)-mellein. Based on these results, novel primers were designed, optimised and tested on the genomic DNA of _D. mutila_, _D. seriata_, _Lasiodiplodia theobromae_, _D. vidmadera_, _N. australe_, _N. luteum_ and _N. parvum_. The resulting PCR products were sequenced and had the highest homology to _D. corticola_ PKSs involved in the synthesis of 6-MSA. However, these preliminary results should be confirmed by further investigations.

To our knowledge, this was the first study to investigate the PMs produced by _Botryosphaeriaceae_ spp. isolated from grapevines in Australia. Insights from this multidisciplinary research will provide knowledge that may assist in elucidating the role of PMs in the pathogenicity, symptom development and plant-pathogen interaction. Future investigation may result in methods being developed to better assist in field diagnosis and control of Botryosphaeria dieback in Australian vineyards.
## List of Abbreviation

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Univariate Analysis of Variance</td>
</tr>
<tr>
<td>BD</td>
<td>Botryosphaeria dieback</td>
</tr>
<tr>
<td>BLASTn</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CAZymes</td>
<td>Carbohydrate-Active enzyme</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>Deuterated Chloroform</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
</tr>
<tr>
<td>COSY</td>
<td>Homonuclear Correlation Spectroscopy Sequence</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyl Trimethyl Ammonium Bromide</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless Enhancement by Polarization Transfer</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECD</td>
<td>Electronic Circular Dicroism</td>
</tr>
<tr>
<td>ED</td>
<td>Eutypa Dieback</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EPSs</td>
<td>Exopolysaccharides</td>
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<tr>
<td>EtOAc</td>
<td>Ethyl Acetate</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>FT-IR</td>
<td>Fourier Transform Infrared</td>
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<tr>
<td>gDNA</td>
<td>genomic Deoxyribonucleic Acid</td>
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<tr>
<td>GTDs</td>
<td>Grapevine trunk diseases</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HR ESIMS</td>
<td>High Resolution Electrospray Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>hrPKs</td>
<td>highly reducing Polyketides synthase</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Correlation</td>
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<tr>
<td>HSTs</td>
<td>Host Selective Toxins</td>
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<tr>
<td>IP</td>
<td>Inoculation Point</td>
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<tr>
<td>i-PrOH</td>
<td>Iso-Propanol</td>
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<tr>
<td>LC/MS-MS</td>
<td>Liquid Chromatography tandem Mass Spectrometry</td>
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LC-QqQ  Liquid Chromatography triple Quadrupole mass spectrometry detector
LSD   Least significant differences
Me$_2$CO  Acetone
MeOH   Methanol
MRM   Multi Reaction Monitoring
MSA   Methyl Salicylic Acid
NHSTs  Non-Host Selective Toxins
NMR   Nuclear Magnetic Resonance
nrPKs  non-reducing Polyketides synthase
NRPS  Non-Ribosomal Peptides
NSW   New South Wales
NWGIC  National Wine and Grape Industry Centre
PCR   Polymerase Chain Reaction
PDA   Potato Destrose Agar
PDA-C  Potato Destrose Agar- Chloramphenicol
PI    Post Inoculation
PKS   Polyketide Synthase
PMs   Phytotoxic Metabolites
pr-PKs Partially Reducing Polyketides Synthase
qPCR  quantitative Polymerase Chain Reaction
RCBD  Randomised Complete Block Design
ROS   Reactive Oxygen Species
SA    South Australia
SDW   Steril Distilled Water
SMs   Secondary Metabolites
TLC   Thin Layer Chromatography
TOF   Time Of Flight
USA   United States of America
UV    Ultraviolet
VCD   Vibrational Circular Dicroism
Chapter 1 – Introduction and Literature Review

1.1 Introduction
Secondary metabolites (SMs) produced by fungal species are in general, not essential for their growth but are believed to be advantageous under certain conditions and in distinct habitats, such as host colonisation for pathogenic fungi. Understanding their role in virulence remains an important challenge for chemists, molecular biologist and plant pathologist. Indeed, SMs produced by fungal pathogens may play an important role in virulence and they may be linked to the symptoms observed on infected plants. Furthermore, another fundamental role played by these metabolites is in the reciprocal crosstalk between plant and fungus (Kusari et al. 2013, Chini et al. 2018, Fonseca et al. 2018). Various studies have shown that these metabolites play an important role in the crosstalk between fungi and their hosts (Dodds and Rathjen 2010, Kusari et al. 2012, Peyraud et al. 2017). For instance, many endophytes could possibly be latent pathogens and they might be influenced by certain intrinsic or environmental factors to express factors that lead to pathogenicity (Kusari et al. 2012). An increasing number of investigations highlighted that certain SMs appear in nature only when specific organisms interact and pathogens may alter plant gene expression and trigger the synthesis of compounds that help resist pathogen attack (Peyraud et al. 2017). Moreover, some metabolites may act as natural agents of competition or as communication signals in the environment and thus are important in certain pathogen–host interactions. Fungal SMs act in different ways and increase the pathogen’s ability to counteract adverse conditions in the host environment, irrespective of whether it is an animal or plant host (Scharf et al. 2014). They can be divided into four main chemical classes: polyketides, terpenoids, shikimic acid derived compounds and non-ribosomal peptides. They usually play a fundamental role in plant-pathogen interaction. However information on their exact mode of action are limited (Pusztahelyi et al. 2015).

Some of the SMs can be classified as fungal phytotoxic metabolites (PMs) and they are usually divided into host-selective toxins (HSTs) and non-host selective (NHSTs) toxins (Pusztahelyi et al. 2015). The PMs produced by pathogenic fungi of crops and forests trees showed different kind of activities, including anticancer, anti-inflammatory, antioxidant, anti-fungal and phytotoxic effects when assayed on different conditions and hosts. Thus, many investigations have been carried out on their isolation, chemical and biological characterisation during the last decades. These metabolites may also be used in agriculture as agents of biological control for weeds or specific plant diseases. (Scharf et al. 2014, Cimmino et al. 2015, Masi et al. 2018b).
Chapter 1

The host-pathogen interaction and how this interaction regulates genes involved in the production of these SMs is another crucial investigation area in plant science. Studying the genes coding for the production of these SMs allows a deeper understanding of their function at the molecular level and their role in the development of plant diseases.

Grapevines are one of the most economically important crops worldwide with approximately 71% of the world grape production being used for wine production. Australia is one of the most important wine producers in the world (Wine Australia 2018). A variety of fungal diseases threaten viticultural regions all over the world, compromising the yield and quality of wine (Halleen et al. 2006, Gessler et al. 2011, Úrbez-Torres 2011). Among them, grapevine trunk diseases (GTDs), caused by one or several xylem-inhabiting fungi, cause a progressive decline in vines, initially causing a loss in productivity and eventually death of the vines (Mondello et al. 2018). Over the past few decades the frequency of symptoms due to GTDs has increased considerably (Gramaje et al. 2018, Mondello et al. 2018). This may be due to better identification methods for the diseases. However the relationship of GTDs with biotic and abiotic stresses, the expression of symptoms and the lack of effective management strategies requires further investigation (Bertsch et al. 2013, Billones-Baaijens et al. 2018, Gramaje et al. 2018, Mondello et al. 2018).

A large number of plant pathologists, molecular biologists and chemists have been investigating different aspects of the pathogenicity, biochemistry and chemical ecology of the fungal species involved in GTDs (Bertsch et al. 2013, Fontaine et al. 2016). To date, up to 133 fungal species belonging to 34 genera have been associated with GTDs worldwide, although Koch’s postulates have not been completed for all of them (Gramaje et al. 2018). The control of GTDs represents a big challenge for winegrowers, nurseries, technicians, and scientists, mainly because of their complexity compared with other grapevine diseases. No grapevine taxa, either cultivated or wild, are known to be resistant to GTDs (Bertsch et al. 2013). The varying symptoms caused by GTDs increases the complexity of accurately identifying them in vineyards, in many cases the disease is recognised only once it has established in the grapevine. Another problem in understanding these diseases is that there are often many pathogens that interact and the infection by one virulent pathogen could lead to a weakened vine that is more vulnerable to infection by other fungi (Fischer and Kassemeyer 2015). These issues clearly add further complexity in linking the symptoms with the causative pathogens. The main trunk diseases that threaten vineyards worldwide are Eutypa dieback (ED), Esca complex and Botryosphaeria dieback (BD).
1.2 Botryosphaeria dieback

The interest in BD has increased substantially over the last decades due to its increasing incidence in vineyards worldwide (Úrbez-Torres 2011, Billones-Baaijens et al. 2018, Gramaje et al. 2018, Mondello et al. 2018). The disease is characterised by external symptoms that include death of the cordons, canes, shoots and buds, the formation of cankers, stunting of shoots, delayed bud burst, bud necrosis and bleached canes (Figure 1.1) (Mondello et al. 2018).

![Figure 1.1. Canker in the trunk of a grapevine and dead cordons and spurs (Photo: Sandra Savocchia).](image)

The internal symptoms in the trunk or canes of declining vines include brown streaking of the wood and wedge-shaped discolorations (van Niekerk et al. 2006, Úrbez-Torres and Gubler 2009, Úrbez-Torres 2011) (Figure 1.2).
BD was thought to be a problem only in older and mature vines (Larignon et al. 2001). However, surveys in Australia, New Zealand and in other regions showed that many species of the Botryosphaeriaceae were frequently isolated from apparently healthy and symptomatic young vines and propagation materials from nurseries and young vineyard (Edwards and Pascoe 2004, Fourie and Halleen 2004; Giménez-Jaime et al. 2006; Aroca et al. 2010; Baaijens 2013; Whitelaw-Weckert et al. 2013). Nonetheless, further studies are required to fully investigate the incidence of BD dieback in young vineyards.

Unlike Esca complex and ED, the appearance of foliar symptoms due to BD varies from region to region. In Europe, foliar symptoms including yellowish-orange (white cultivars) or wine red (red cultivars) discolourations were first reported by Larignon et al. (2001). However, in Australia, where ED and BD are considered the two most important grapevine trunk diseases (Billones-Baaijens et al. 2018), no foliar symptoms have been reported to date (Pitt et al. 2013a). The production and subsequent translocation of PMs produced by the fungal pathogens are considered to be principally responsible for the appearance of foliar symptoms. However, the erratic and unpredictable nature of their appearance may be due to more complex interactions between the plant, fungus and environment that require more in-depth study.

1.2.1 Distribution of Botryosphaeriaceae spp.
Species in the Botryosphaeriaceae have a cosmopolitan distribution and occur on a wide range of annual and perennial hosts including grapevines (Crous et al. 2006, Slippers and Wingfield 2007, Hyde et al. 2013). Molecular approaches provide improved accuracy, reliability and reproducibility in identifying the species. Despite the importance attributed to the pathogenic botryosphaeriaceous taxa, few molecular studies have been conducted to improve the understanding of the biology and
ecology of these species (Chethana et al. 2016). Members of the *Botryosphaeriaceae* have been described as endophytes and as latent pathogens causing serious diseases (Slippers and Wingfield, 2007; Sakalidis, 2011; Yan et al., 2013). Biochemical and genetic responses caused by external stimuli resulting from changing environmental conditions inside hosts (changes in host behaviour or microbial equilibrium) or outside hosts (changes in climate or extreme environmental events) triggers the fungi to change their lifestyles from endophytic to pathogenic. Due to this transition, these fungi can be regarded as plant opportunistic fungal pathogens since their pathogenic nature may appear when induced by environmental factors or when the vigour of plants becomes weak (Kusari et al. 2012, Chethana et al. 2016). The endophytic phase of some fungi involved in GTDs is well-recognised (Mugnai et al. 1999, Mostert et al. 2000). However, the status of *Botryosphaeriaceae* species as endophytes in grapevines is still unclear. Differences in host susceptibility, wounding, virulence of pathogen, adverse environmental conditions such as drought further amplify the pathogenicity of the *Botryosphaeriaceae* (Úrbez-Torres et al. 2011).

*Botryosphaeriaceae* species occur in different grape growing regions of Africa, Asia, Australia, Central America, Europe and South America (Úrbez-Torres et al. 2006, Pitt et al. 2010, Úrbez-Torres et al. 2012, Linaldeddu et al. 2015). Different species of *Botryosphaeriaceae* belonging to the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryosphaeria* and *Spencermartinsia* have been reported to be associated with BD of grapevines worldwide. The isolation of *S. westrale*, *S. plurivora*, *D. neclivorem*, *D. vineagemmeae* and *Dothiorella sp.* from perennial cankers has brought the total number of Botryosphaeriaceous species isolated from grapevines worldwide to 26 (Pitt et al. 2015, Comont et al. 2016, Gramaje et al. 2018). The species infecting grapevines can be classified according to their virulence and can be divided into three different groups including the highly virulent species, *Lasiodiplodia spp.* and *Neufusicoccum spp.*, intermediately virulent *B. dothidea* and *Diplodia spp.*, while *Dothiorella spp.* and *S. viticola* are weakly virulent (Úrbez-Torres (2011). Furthermore, the conidia of *D. seriata* showed the greatest germination percentage and optimum mycelial growth under a wide range of temperatures (10-40°C) which may explain why this species is the most cosmopolitan botryosphaeriaceous fungus infecting grapevines (Úrbez-Torres 2011).

With a particular focus on *Botryosphaeriaceae* spp. found in Australia, during a survey of more than 91 vineyards throughout southern New South Wales (NSW) and South Australia (SA), eight different species including *D. seriata*, *D. mutila*, *L. theobromae*, *N. parvum*, *N. australe*, *B. dothidea*, *S. viticola* and *Do. vidmadera* were isolated and their distribution varied according to geography and climate
Species of Diplodia and Dothiorella, characterised by thick-walled, pigmented conidia were the most prevalent and were distributed widely throughout both NSW and SA while species of Neofusicoccum were isolated less frequently and displayed more limited geographic ranges. Furthermore, Lasiodiplodia spp. were recovered, from Western Australia and the northern most region of NSW (Billones-Baaijens et al. 2018). D. seriata, which accounted for almost 80% of the total number of Botryosphaeriaceae isolates collected was the most common isolated species confirming its cosmopolitan nature. Furthermore, taxonomic and genetic investigations on Dothiorella and Spencermartinsia spp. isolated in Australian vineyards resulted in the reclassification of Do. iberica to Do. vidmadera and the first report of S. plurivola (Pitt et al. 2013b, Pitt et al. 2015). Finally, with the isolation of N. ribis (Wunderlich et al. 2011) the number of Botryosphaeriaceae species isolated in Australian vineyard increased to 11.

N. parvum along with N. australae and L. theobromae produced the longest lesions and were among the most virulent of the tested species (Pitt et al. 2013a). Furthermore, small differences were recorded between B. dothidea and Diplodia spp., S. viticola and Do. vidmadera, thus all species were considered ‘moderately’ pathogenic in comparison to the more aggressive species (Pitt et al. 2013a). However, for S. viticola and Do. vidmadera this is in contrast with previous reports that classified these species as ‘weakly’ pathogenic (Ürbez-Torres 2011). These findings highlights that the pathogenicity of Botryosphaeriaceae spp. can be dependent on environmental factors and can vary from region to region.

1.3 Phytotoxic metabolites produced by Botryosphaeriaceae spp. involved in GTDs.

Many studies have been carried out to investigate the virulence factors that assist fungi to invade and colonise important economic crops such as grapevines. Carbohydrate-active enzymes (CAZymes), peroxidases, effector proteins, cytochrome P450s; cellular transporters; and secondary metabolism, including toxin production are only some of the studied virulence factors (Bielska et al. 2014, Pusztahelyi et al. 2015, Geoghegan et al. 2017).

In the last decades, several studies have been conducted on the isolation and characterisation of PMs produced in vitro by pathogens involved in GTDs. Many of these metabolites have been chemically-characterised and tested for their toxicity on grapevines and non-host plants, and different reviews and perspectives on these topic can be found in the literature (Andolfi et al. 2011, Masi et al. 2018a). Interestingly, some of these compounds are typical for a specific pathogen. For example, eutypine (1 Figure 1.3) is a typical fungal PM produced by Eutypa lata the causal agent of ED (Tey-Rulh et al.,
1991), while *Phaeacremunium minimum* and *Phaeomoniella chlamydospora*, the two main tracheotomy fungi involved in the Esca complex, produce two phytotoxic naphthalenone pentaketides: scytalone and isosclerone (2,3 Figure 1.3) (Tabacchi et al. 2000).

![Figure 1.3. Structure of eutypine (1), scytalone (2) and isosclerone (3).](image)

Studies on the phytotoxicity of PMs produced by *Botryosphaeriaceae* spp. involved in GTDs were first reported by Martos et al. (2008). The production of PMs in liquid culture by five species isolated from declining grapevines in Spain (*B. dothidea, D. seriata, Do. viticola, N. luteum* and *N. parvum*) was assessed. All fungi produced hydrophilic high-molecular weight compounds, in particular exopolysaccarides (EPSs) with phytotoxic properties. In addition, the preliminary analysis of the organic extracts of the culture filtrates showed that *N. luteum* and *N. parvum* produced lipophilic low molecular weight phytotoxins (Martos et al. 2008).

Among the 26 *Botryosphaeriaceae* spp. infecting grapevines worldwide, *N. parvum* and *D. seriata* are, respectively, the most aggressive and the most widespread around the world (Úrbez-Torres 2011), and therefore they are the most studied species for the production of PMs. Several phenolic dihydroisocoumarins, such as *(R)-mellein, (3R,4R)-and (3R,4S)-4-hydroxy melleins and 5-hydroxymellein (4-7, Figure 1.4) were identified from the organic extract of the culture filtrate of *D. seriata* (Djoukeng et al. 2009). Furthermore, they identified an unknown mellein characterised as *(3R,4R)-4,7-dihydroxy mellein (8, Figure 1.4).

In the case of *N. parvum*, four phytotoxic metabolites were isolated from organic extracts and identified by spectroscopic and physical examination as the isosclerone (3), tyrosol (9, Figure 1.4) and the previously reported 4-hydroxy-mellein cis and trans mellein (5, 6 Figure 1.4) (Evidente et al. 2010). Liquid chromatography-diode array screening of the organic extract of the cultures of 13 isolates of *N. parvum* resulted in 13 compounds belonging to four different chemical families being identified through spectroscopic analyses and by comparison to previously published literature as
(R)-mellein and its derivatives belong to the class of isocoumarines. This class of natural compounds is well known to have a wide range of biological activity ranging from antimicrobial to anticancer and anti HIV (Saeed 2016). In particular, (R)-mellein, has shown antibacterial, phytotoxic, larvicidal, fungicidal, HCV protease enzyme and prostaglandin synthesis inhibitor properties (Saeed 2016). Moreover, (R)-mellein is considered to be a typical PM produced by Botryosphaeriaceae spp. involved in GTD and is phytotoxic to grapevine leaves at different concentrations (Djoukeng et al. 2009, Evidente et al., 2010).

Ramirez-Suero et al. (2014) showed that (R)-mellein only cannot explain the toxicity of the extracellular organic extract of D. seriata and N. parvum. Purified (R)-mellein was added to the culture medium of calli, but only a delayed necrosis and a lower-level expression of defense genes were observed. In addition, the extracellular compounds from N. parvum appeared to be more toxic than those produced by D. seriata. Finally, the authors suggested that it is possible that the pathogenicity of these two fungi depends on a synergistic action between the secretion of other types of PMs, such as derivatives of mellein or high molecular weight phytotoxins such as polypeptides or EPSs (Ramírez-Suero et al. 2014).

The extracellular EPSs produced by an isolate of N. parvum isolated from infected grapevine wood in a vineyard in Spain were biologically and chemically characterised by Cimmino et al. (2016). The EPS was characterised as a mannan having a backbone consisting of α (1→6)-linked mannopyranose units almost all branched at the 2 position, whereby the arms were composed of 2- and/or 3- linked units. Phytotoxic activity was observed when assayed on grapevine leaves. However, the three replicates of each tested concentration developed symptoms at different times and the differences in the type of symptoms induced were observed, therefore, a conclusion could not be drawn (Cimmino et al. 2016).
More recently, purified secreted proteins by *N. parvum* and *D. seriata* were assayed on suspension cells of two different *Vitis* genotypes (*V. rupestris* and *V. vinifera* cv. Gewurztraminer) with putative varying susceptibility to BD (Stempień et al., 2018). The *Vitis* cells were able to detect secreted proteins produced by Botryosphaeriaceae resulting in a rapid alkalinisation of the extracellular medium and the production of reactive oxygen species. *V. rupestris* is characterised by higher medium alkalinisation, cell death, and more intense induction of pathogenesis-related genes, whereas *V. vinifera* cv. Gewurztraminer shows a higher production of the antifungal, δ-viniferin. The results further suggested that even if the grapevine is able to react rapidly to trunk disease pathogens, the defense responses are most likely not strong enough to restrict the growth of the pathogen. However, further studies are required to determine the sequences of the secreted protein and their mode of action (Stempień et al. 2018).

*N. australe* involved in grapevine decline in Sardinia produced a new cyclohexenone oxide, namely, cyclobotryoxide (20, Figure 1.4), that was isolated together with 3-methylcatechol (21, Figure 1.4) and tyrosol. Cyclobotryoxide appeared to be the most active metabolite in the different bioassays performed (Andolfi et al. 2012).
Figure 1.4. Structure of secondary metabolites produced by *Botryosphaeriaceae* spp. involved in grapevine trunk diseases. (R)-mellein (4), (3R,4R)- and (3R,4S)-4-hydroxy melleins (5, 6), 5-hydroxymellein (7), (3R,4R)-4,7- dihydroxy mellein (8), tyrosol (9), (+)-terremutin (10), (+)-terremutin hydrate (11), (+)-epi-sphaeropsidone (12), (-)-4-chloro-terremutin hydrate (13), (+)-4—hydroxysuccinate-terremutin hydrate (14), (6R,7R)-asperlin (15), (6R,7S)-dia-asperlin (16), (R)-3-hydroxymellein(17), 6-methyl-salicylic acid (18), 2-hydroxypropyl salicylic acid (19), cyclobotryoxide (20), 3-methylcatechol (21).

Several endophytic and pathogenic fungi are capable of producing PMs that are also biosynthesised by their host plants (Kusari et al. 2013). In the case of the *Botryosphaeriaceae* spp. involved in GTDs, *Lasiodiplodia* spp. are capable of produce jasmonic acid (22, Figure 1.5), a known plant hormone and some of its derivatives when grown in *in vitro* condition. Jasmonic acid, its methyl ester (23, Figure 1.5) and Lasiojasmonate A-C (24-26, Figure 1.5) were isolated from the grapevine pathogen *L. mediterannea* (Andolfi et al. 2014). The mode of action of jasmonic acid and lasiojasmonate A as fungal phytotoxins was investigated and the results suggested that the production of jasmonic acid derivative such as lasiojasmonate A occurs during the late stages of infection to induce plant jasmonic
acid responses such as cell death and to facilitate fungal infection (Chini et al. 2018; Reveglia et al. 2018a). More recently, two novel compounds identified as lasiolactol A and B were isolated and characterised (27, 28 Figure 1.5) from another strain of *L. mediterranea* isolated from grapevines in Sicily. These two novel molecules were isolated together with botryosphaeriodiplodin (29, Figure 1.5), (5R)-5-hydroxylasiodiplodin (30, Figure 1.5) and (3S,4R,5R)-4-hydroxymethyl-3,5-dimethyl dihydro-2-furanone (31, Figure 1.5), all previously characterised secondary metabolites (Andolfi et al. 2016).

Finally, LC-MS analysis of *L. brasiliense*, *L. crassispora*, *L. jatrophicola*, *L. pseudotheobromae* isolated from grapevines with BD in Brazil produced jasmonic acid, and *L. brasiliense* also produced (3R,4S)-4-hydroxymellein (Cimmino et al. 2017). *L. euphorbicola* produced (R)-mellein, (3R,4R)- and (3R,4S)-4-hydroxymellein and tyrosol, while *L. hormozganensis* synthesised tyrosol and p-hydroxybenzoic acid (32, Figure 1.5). All these compounds showed phytotoxicity when assayed on grapevine leaves (Cimmino et al. 2017).

![Figure 1.5. Structure of jasmonic acid (22), jasmonic acid methyl ester (23), lasiojasmonate A (24), lasiojasmonates B and C (25, 26), lasiolactol A and B (27, 28), botryosphaeriodiplodin (29), (5R)-5-hydroxylasiodiplodin (30), (3S,4R,5R)-4-hydroxymethyl-3,5-dimethyl dihydro-2-furanone (31), p-hydroxybenzoic acid (32).](image-url)
1.4 The role of phytotoxic metabolites in grapevine tissues infected with GTDs

Although several studies have been carried out on the isolation and characterisation of PMs produced by GTD pathogens, only a few have tried to investigate their role in pathogenicity and symptom expression (Tabacchi et al. 2000, Rolshausen et al. 2008). The lack of studies may be due to the complexity of GTDs and the pathogens involved. Further studies are needed to take into account: plant-pathogen interaction, pathogen-pathogen interaction, environmental factors, etc. (Bertsch et al. 2013, Fontaine et al. 2016). In vitro experiments or experiments based on artificially inoculated vines in the glasshouse are often useful, although these can only provide a partial view of the role of PMs as virulence factors. The production of PMs may be related to the expression of foliar symptoms. The current hypothesis is that they are produced by the pathogen in the trunk and they can migrate without being catabolised or, at least, without being entirely detoxified to the leaves where they cause the common observed symptoms (Figure 1.6). This hypothesis was proposed for the first time by Tey-Rulh et al. (1991) to explain the foliar symptoms produced by ED. When the PMs were isolated and identified from the pathogens involved in Esca and BD, this hypothesis was also extended to these two GTDs (Andolfi et al. 2011). However, further investigations are needed to support this hypothesis and to correlate the production of PMs with foliar symptoms.

Figure 1.6. Schematic representation of the hypothesis that fungal phytotoxins are able to translocate within a vine.

*P. minimum* and *P. chlamydospora* are capable of producing different naphthoquinones but little information is known about the function of these metabolites in vine cells or tissues (Tabacchi et al. 2000, Andolfi et al. 2011). Their activity might be related to their oxidant property, especially their interaction with reactive oxygen species (ROS) produced by the plant during the defense response (Medentsev and Akimenko 1998, De Gara et al. 2003). Thus, the presence of the typical tiger-stripe
symptoms on leaves from grapevines infected with Esca may be related to physiological changes caused by toxic metabolites produced by the causal pathogens in the trunk. In this case, these changes would be a response of the vine to the disease (Andolfi et al. 2011).

Several efforts have been made to identify eutypine from different tissues showing ED symptoms. In vitro cultures of *E. lata* isolates were able to produce various SMs of which eutypine was the main metabolite, however HPLC analysis of extracts from wood, shoots and leaves exhibiting symptoms of dieback failed to show the presence of any metabolites (Mahoney et al. 2005). Micropropagated grapevine plantlets treated with purified or crude culture filtrates from nine isolates of *E. lata* grown on malt yeast broth resulted in various SMs being identified. However, no single compound was detected consistently (Lardner et al. 2006). A derivative of eutypine, named eutypinol was detected in micropropagated grapevine plantlets inoculated with mycelium of *E. lata*, but no PMs were detected in the sap of vines which had been artificially inoculated with the pathogen (Lardner et al. 2006).

The reasons for the unsuccessful detection of eutypine remain unclear. However, it is possible that following their entry into grapevine tissue, such metabolites are rapidly broken down into compounds that cannot be detected by HPLC (Mahoney et al. 2005). Alternatively, the translocation of metabolites does not occur because the compounds are sufficiently reactive to damage plant tissues in the proximity of fungal infection, reacting in such a way that they are bound irreversibly. Finally, the authors of these studies suggested that as phenolic compounds, the metabolites would be susceptible to oxidative polymerization by plant phenol oxidases, possibly accounting for the dark, wedge-shaped areas typical of *E. lata* infection (Mahoney et al. 2005, Lardner et al. 2006, Rolshausen et al. 2008).

The relationship between the botryosphaeriaceous fungi found in the diseased wood and the expression of foliar symptoms observed in some cultivars in the northern hemisphere is still not well understood. A recent investigation by Reis et al. (2016) resulted in the development of a simple model system to reproduce the foliar symptoms caused by *D. seriata* and *N. parvum* in order to better characterise fungal pathogenicity and to determine the mechanisms involved in symptom development. The experiment was repeated four times from 2011 to 2014 and eight months after inoculation, the percentage of plants showing foliar symptoms was greater for the *N. parvum* treatments than for the *D. seriata* treatments. This study is the first to report the reproduction of foliar symptoms with a frequency close to those observed in the vineyard. Furthermore, the authors
monitored the expression of plant stress-related response genes and observed an upregulation of PR6 (PR6, chitinase, and b-1,3-glucanase) in the leaves. However, these data together with other investigations (Ramírez-Suero et al. 2014, Spagnolo et al. 2014, Bellée et al. 2017) suggest that the responses produced by the plant are inefficient against these pathogens therefore overcoming the plants natural defense. Understanding the mechanisms involved in the infection by *Botryosphaeriaceae* in grapevine is important to develop strategies that limit the spread of the disease.

The infected trunk wood and green shoots from four grapevines (cv. Chardonnay; Avize, Epernay, Champagne-Ardenne region, France and Mourvèdre; Rodilhan, Nîmes, Languedoc Roussillon region, France) showing Esca and/or BD foliar symptoms were analysed to determine whether the common PMs identified in *in vitro* cultures of *N. parvum* were also produced in planta (Abou-Mansour et al. 2015). The analysis was performed for (R)-mellein and (+)-terremutin, which were considered representatives of two of the isolated chemical families, through HPLC-DAD-MSn using organic extracts from powdered wood. Both PMs were detected in the extracts of brown-striped and black-streaked wood samples. This is the only report of PMs produced by *N. parvum* in grapevines showing Esca or BD in vineyards (Abou-Mansour et al. 2015). However, as also described by the authors, the small number of samples analysed and the lack of information on which pathogens were present in the infected analysed wood does not allow a definitive conclusion to be drawn on the role of PMs in the disease. Furthermore, conclusions cannot be drawn on whether the PMs are translocated to the leaves resulting in foliar symptoms, or whether their toxic function is expressed in the trunk.

### 1.5 Genetic basis of the pathogenicity and virulence of *Botryosphaeriaceae* spp.

Pathogenicity genes are defined as genes necessary for disease development but not essential for a pathogen to complete its life cycle (Cacho et al. 2015). In addition, virulence genes also have a role in pathogenicity (Idnurm and Howlett 2001). Natural selection is the major force behind the differences in gene family size between species, and therefore focusing on families with faster rates of gene gain can help identify functions that may be associated with host adaptation pathogenicity or virulence.

The production of SMs by fungi might have a role in host interaction and in advancing the infection. Studying the pathogenicity genes in an ecological context can provide important clues to their functions in the life cycle of the pathogens and their role during the infection process (Idnurm and Howlett 2001, Lim et al. 2012). Comparative genomics and transcriptomics are important tools that could assist in the identification of the complete gene set for the target pathway of SMs. Several SMs
gene cluster prediction software programs have also been developed which can aid this process considerably. Comparative genomics can also be utilised for identifying the biosynthetic gene clusters of a target group of SMs bearing structural similarities (Zhao et al. 2013, Peyraud et al. 2017). For instance, in a recent study, comparative genome and transcriptome analyses enabled the identification of extensive structural variation on the virulence functions in *P. minimum* (Massonnet et al. 2018a). The *P. minimum* genome was enriched in clusters associated with secondary metabolism and the authors hypothesised that acquisition or loss of secondary metabolism functions has an adaptive effect on fitness (Massonnet et al. 2018a). The absence of whole genome sequences slows down the identification of these target genes.

The recent availability of next-generation RNA-Seq technologies has revolutionised transcriptomic profiling and with ready access to microbial genome sequencing enabled by next-generation sequencing technologies, genome sampling and re-sequencing is becoming a routine. Secondary metabolite gene clusters are flanked by synthetic blocks containing highly-conserved core genes. Fungus usually have 20-50 SM genes and ecologically relevant SM gene clusters and their production in fungi is highly regulated and often in response to specific biotic factors and environmental perturbations. For plant pathogens, these transcriptomic tools can be used to probe the expression of SM gene clusters at various stages of infection (Yaegashi et al. 2014, Cacho et al. 2015). However, as suggested by the Chooi and Solomon (2014), to obtain deeper insights into the bio-ecological functions of these SM gene clusters, the encoded secondary metabolite molecules have to be first identified and chemically characterised.

The biosynthesis of PKS in fungi has been studied intensively (Cox 2007). A recent example is the investigation carried out on the PR-PKS gene, SNOG_00477 (SN477), present in the wheat pathogen *Parastagonospora nodorum* (Chooi et al. 2015). SN477 was the only enzyme required for the production of (R)-mellein and this is the first and only identification of a fungal PKS responsible for the synthesis of this phytotoxic secondary metabolite (Chooi et al. 2015).

Despite the importance attributed to the pathogenic botryosphaeriaceous taxa, few molecular studies have been conducted to improve the understanding of the biology and ecology of these species. In general, *Botryosphaeriaceae* species are highly diverse and this may be a reason for their success in adapting to different environmental conditions (Slippers and Wingfield 2007). However, low genetic variability is reported from many geographical locations for *Botryosphaeria, Diplodia* and *Lasiodiplodia* species (Chethana et al. 2016).
Studies focused on genetic diversity and pathogenicity of *Botryosphaeriaceae* species have demonstrated variable levels of virulence between isolates. *N. parvum, N. luteum, L. theobromae, N. australe, D. mutila and D. seriata* were all pathogenic and the most prevalent *Botryosphaeriaceae* species (Úrbez-Torres 2011, Linaldeddu et al. 2015). These species were also highly variable in their pathogenicity and these observations have led to the initiation of further molecular studies to demonstrate correlations between genetic diversity, pathogenicity and virulence (Úrbez-Torres and Gubler 2009, Baskarathevan et al. 2012, Billones-Baaijens et al. 2013, Pitt et al. 2013a). All these pathogens have gene clusters involved in the production of molecular components such as extracellular compounds that play important roles in nutrient release or uptake or in the virulence (Morales-Cruz et al. 2015) To date, there are no documented candidate sequences for any of the potential virulence factors associated with trunk diseases. The proteomes of six isolates of *D. seriata* and *N. parvum* were annotated with a focus on key functions likely associated with pathogenesis and virulence: wood degradation and host colonization enzymes such as carbohydrate-active enzyme (CAZymes), peroxidases, cytochrome P450s, cellular transporters and secondary metabolism, with a particular focus on toxin production (Morales-Cruz et al. 2015). Comparative analysis from the same study also revealed a broad range in the number of members of gene families with potential virulence functions. Genes involved in the same secondary metabolic pathway are often physically clustered on fungal chromosomes. These gene clusters typically comprise a central biosynthetic gene surrounded by other genes encoding transporters and other enzymes involved in post-synthesis modification. Auxiliary activity CAZymes AA3, extracellular hemoflavoenzymes, were particular abundant in *N. parvum* and AA1 genes that encode for multicopper oxidases, including laccases, were particularly abundant in *N. parvum* and *D. seriata* (Morales-Cruz et al. 2015). None of the genes involved in the synthesis of PMs have been identified to date. In fungi, genes involved in the synthesis and transport of SMs are typically clustered together with the gene coding for the key biosynthetic enzyme. Many secondary metabolic clusters were observed in the ascomycete trunk pathogens mostly associated with the synthesis of PKs and fatty acid-derived compounds, terpenes, and non-ribosomal peptides and amino acid derived compounds (NRPS) (Morales-Cruz et al. 2015). A remarkable expansion of genes associated with non-ribosomal peptides and amino acid derived compounds was observed for *N. parvum*. Furthermore, the gene counts of these secondary metabolite clusters were different for *N. parvum* and *D. seriata* and this reflect their different rates of wood colonisation and the more rapid rate of wood necrosis (Morales-Cruz et al. 2015).
A more recent study on the virulence factors of *N. parvum* revealed that physically clustered genes coding for putative virulence functions were induced depending on the stage of plant infection (Massonnet et al. 2018b). Co-expressed gene clusters were significantly enriched suggesting that dynamic co-regulation of transcriptional networks contributes to different aspects of *N. parvum* virulence. In most of the co-expressed clusters, all genes shared at least a common pattern in their promoter region, indicative of co-regulation by the same transcription factor. Co-expression analysis also identified chromatin regulators with correlated expression with inducible clusters of virulence factors, suggesting a complex regulation of the virulence repertoire of *N. parvum* (Massonnet et al. 2018b).

In summary, the whole genome data of *Botryosphaeriaceae* fungi, and consequently deeper molecular studies, might assist in the discovery of other molecules responsible for fungal-plant interactions. Identification of these molecules is important to understand: (i) the interactions with the host, (ii) the interaction with the others microorganisms that compose the microbiome of the plant, (iii) insight on the lifestyle of the pathogens. This knowledge could assist in designing strategies to alter the balance of these interactions to decrease pathogenicity.

1.6 Summary and research objectives

Of all the GTDs, BD appears to be the most suitable as a model for *in planta* experimentation and to understand the connection between the symptoms observed in the vineyard and the role of PMs in the expression of these symptoms. The reasons for this include: (i) the disease is caused by only one family of pathogenic fungi; (ii) the relatively large number of species (at least 26) involved in the disease permits a comparison in pathogenicity and virulence between isolates and species; (iii) the typical phytotoxic SMs produced by these pathogens are well-known, chemically characterised and for some of them their toxicity *in vitro* is well-studied.

Thus, the main goal of further investigation should be to understand the role of these PMs on disease expression, their toxicity against other hosts and their biological functions in the various stages of the lifecycle of the fungi. This objective might be better facilitated by *in planta* studies. One of the limits in achieving this objective in the past decade has been due to the low distribution and high cost of sensitive analytical techniques. However, it is clear that with the development of more sensitive analytical chemistry equipment and more efficient extraction protocols the detection of these metabolites, even in traces, in infected wood, has become possible. Indeed, the detection of (R)-mellein in symptomatic wood reported by Abou-Mansour et al. (2015) represents a good starting
point for further and more detailed investigations. Furthermore, plant metabolomics can also be a powerful tool in the study of metabolite accumulation and metabolic regulation. Metabolomics offers a better understanding of complex biological systems, different studies suggesting that the combination of metabolite profiling and non-targeted metabolic approaches can assist in disease marker selection, which are then combined with host transcriptomic data (Schauer and Fernie 2006, Vasilev et al. 2016). However, because the specific responses depend not only on the plant species but also on other factors such as the interacting microorganisms, the type of interaction, and the plant organs involved, evaluating the simultaneous impact of different parameters on metabolic pathways is a challenging task and this limits the capability to extrapolate the results obtained from a particular plant-microbe interaction to another. Nevertheless, in spite of these setbacks, some broad conclusions when working with related plant species can be made (Romero et al. 2017). Comprehensive elucidation of the molecular, biochemical and physiological processes associated with plant–pathogen interactions will provide a better understanding of GTDs and may assist in the development of novel and efficient strategies to management the disease in the vineyard.

This PhD project aims to:

(i) Isolate and biologically and chemically characterise the PMs produced by different species of *Botryosphaeriaceae* from Australian vineyards.

(ii) Investigate the production of PMs *in planta* and to develop an LC/MS-MS protocol to analyse naturally infected and artificially infected vines.

(iii) Genetics of PMs production by *Botryosphaeriaceae* species

This PhD study has resulted in four published peer-reviewed journal articles and an additional manuscript that is being drafted for publication. Preliminary results leading to these articles have also been presented at four conferences and workshops (Appendix A).
Chapter 2 – Phytotoxic metabolites from Australian Botryosphaeriaceae spp.: chemical and biological characterisation

2.1 Introduction

As described in Chapter 1, there is a need to investigate the PMs produced by Botryosphaeriaceae species involved in BD to clarify their role in the expression of symptoms and virulence. Prior to this investigation there was no literature available on the PMs produced by Australian species of Botryosphaeriaceae. In this Chapter, the results of a preliminary investigation on the production of PMs by 24 isolates of the nine most virulent and widespread species of Botryosphaeriaceae in Australia are reported. In addition, previously reported PMs were isolated and identified from the culture filtrates of D. mutila DAR79137, D. seriata H141a, N. australe DAR79506 and N. luteum DAR81016. Furthermore, PMs isolated and characterised for the first time from S. viticola DAR78870 and Dothiorella vidmadera DAR78993 are reported in this Chapter as two peer-reviewed journal articles.

Finally, further investigations on culture filtrates of D. mutila DAR79137 led to the isolation and characterisation of two novel compounds and are reported in a peer reviewed journal article. The 1D and 2D $^1$H-NMR and $^{13}$C-NMR spectra of the novel PMs described in the manuscripts and the $^1$H NMR of the already known compounds are reported in Appendix B. The commercial suppliers of the reagents used in all the experiments conducted in this thesis are provided in Table 2.1.
Table 2.1. List of the commercial suppliers of reagents used to conduct the experiments.

<table>
<thead>
<tr>
<th>Reagent</th>
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</thead>
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<td>CDCl$_3$</td>
<td>Sigma-Aldrich, USA</td>
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<tr>
<td>CHCl$_3$</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>MeOH</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>$n$-hexane</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Water 0.1% formic acid</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>ACN</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Phenomenex LUNA</td>
<td>Torrance, USA</td>
</tr>
<tr>
<td>Analytical and preparative TLCs</td>
<td>Merck, Germany</td>
</tr>
<tr>
<td>Silica gel</td>
<td>Merck, Germany</td>
</tr>
<tr>
<td>PrepMan Ultra</td>
<td>Applied Biosystems, U.K.</td>
</tr>
<tr>
<td>CTAB</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Sigma Aldrich, USA</td>
</tr>
<tr>
<td>RNAse A</td>
<td>Qiagen, USA</td>
</tr>
<tr>
<td>Qiagen DNeasy Plant DNA extraction kit</td>
<td>Qiagen, USA</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>Bioline, USA</td>
</tr>
<tr>
<td>TaqRed DNA polymerase</td>
<td>Bioline, UK</td>
</tr>
<tr>
<td>Agarose</td>
<td>Bioline, UK</td>
</tr>
<tr>
<td>GelRed™ nucleic acid gel stain</td>
<td>Biotium, Hayward, CA</td>
</tr>
<tr>
<td>FavorPrep Gel/PCR purification kit</td>
<td>Favorgen Biotech Corp, USA</td>
</tr>
</tbody>
</table>
2.2 Research Paper – Phytotoxic Metabolites by Nine Species of *Botryosphaericeae* Involved in Grapevine Dieback in Australia and Identification of Those Produced by *Diplodia mutila, Diplodia seriata, Neofusicoccum australe* and *Neofusicoccum luteum*.


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Phytotoxic metabolites by nine species of Botryosphaeriaceae involved in grapevine dieback in Australia and identification of those produced by *Diplodia mutila*, *Diplodia seriata*, *Neofusicoccum australae* and *Neofusicoccum luteum*

Pierluigi Reveglia, Sandra Savocchia, Regina Billones-Baaijens, Marco Masi, Alessio Cimmino & Antonio Evidente

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To link to this article: https://doi.org/10.1080/14786419.2018.1497631
Phytotoxic metabolites by nine species of Botryosphaeriaceae involved in grapevine dieback in Australia and identification of those produced by Diplodia mutila, Diplodia seriata, Neofusicoccum australe and Neofusicoccum luteum

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ABSTRACT
Botryosphaeria dieback is one of the main trunk diseases of grapevine caused by several species of Botryosphaeriaceae. Twenty-four fungal isolates representing the eight most widespread and most virulent Botryosphaeriaceae were tested for their ability to produce phytotoxic metabolites. The chromatographic profiles of their culture filtrates organic extracts showed the ability of all isolates to produce several and different metabolites. When tested on grapevine leaves and tomato cuttings the organic extracts phytotoxicity varied among isolates and species. To our knowledge, this is the first study on phytotoxic compounds produced by Botryosphaeriaceae species found in Australian vineyards. The phytotoxic metabolites produced by Diplodia seriata, Diplodia mutila, Neofusicoccum australe and, for the first time, by Neofusicoccum luteum were isolated and chemically identified essentially by spectroscopic methods.

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Supplemental data for this article can be accessed at https://doi.org/10.1080/14786419.2018.1497631.
1. Introduction

Among the biotic stress agents that induce serious disease to grapevine (*Vitis vinifera* L.), fungi are the most widespread and are present in many regions where grapevines are cultivated. Some of the most important diseases that cause yield and economic losses in vineyards worldwide are grapevine trunk diseases (GTDs). GTDs are caused by various pathogenic fungi including *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum*, two well-known tracheomycotic fungi associated with other *Phaeoacremonium* species in the esca disease complex. *Eutypa lata*, the casual agent of Eutypa dieback has been reported as a vascular wood pathogen and also produces phytotoxic metabolites. Botryosphaeria dieback is considered a serious problem of grapevines worldwide, including Australia. This disease is caused by several Botryosphaeriaceae species producing cankers, dieback and eventually the death of vines. Botryosphaeriaceae have been associated with grapevine decline symptoms worldwide and several species have also been associated with dieback and the production of wood canker (Pitt et al. 2010; Úrbez-Torres et al. 2012; Andolfi et al. 2011; Abou-Mansour et al. 2015; Cimmino et al. 2017).

In Europe, this disease is also associated with foliar symptoms including yellowish-orange spots on white cultivars or wine-red spots on red cultivars (Larignon et al. 2001; Masi et al. 2018). These symptoms have not been reported in Australian vineyards to date (Pitt et al. 2010), but a wedge-shaped brown lesions in the cross section of the trunk and dieback of the cordon were observed (Figure S1). Many foliar symptoms are usually associated with the production of phytotoxic metabolites belonging to different classes of naturally occurring compounds such as aromatic compounds, isocoumarins, jasmonates, naphthalenones polyketides and phenols (Masi et al. 2018). Thus, there is a need to investigate the role of phytotoxins in the symptom expression of pathogens associated with Botryosphaeria dieback.

Recently, phytotoxins produced by *Dothiorella vidmadera* L5 and *Spencermartinsia viticola* L19 were isolated and characterized (Reveglia, Baaijens-Billones et al. 2018; Reveglia, Savocchia et al. 2018).

This manuscript reports the investigation of the production of phytotoxins from the most widespread and virulent Botryosphaeriaceae species associated with Botryosphaeria dieback in Australia (Pitt et al. 2010, 2013).

2. Results and discussion

Four isolates of *Diplodia seriata*, two isolates of *D. mutila*, three isolates each of *Botryosphaeria dothidea*, *Neofusicoccum australe*, *N. luteum*, *N. parvum* and *S. viticola*, two isolates of *D. vidmadera* and one of *Dothiorella* sp. were screened for their ability to produce phytotoxins *in vitro*. All the isolates were grown at 14 days and 21 days. Considering the amount of organic extracts and the phytotoxicity, the optimal grown condition to produce secondary metabolites was at 14 days for all the isolates and this was chosen in this work. The culture filtrates of all isolates were assayed on grapevine leaves (Table 1). Weak and moderate phytotoxicity were observed for all culture filtrates of *B. dothidea*, *D. seriata* and *N. parvum* which resulted in slight wilting, moderate wilting and necrosis. The culture filtrates of *D. mutila*, *D. vidmadera*, *N. australe*,
Table 1. Level of toxicity of secondary metabolites produced by culture filtrates and organic extracts from Botryosphaeriaceae spp. 28 h after treatment on tomato cuttings cv. Grouse and leaves of Vitis vinifera cv. Shiraz.

<table>
<thead>
<tr>
<th>Botryosphaeria spp.</th>
<th>Isolate</th>
<th>pH of the culture filtrate</th>
<th>Tomato cuttings</th>
<th>Grapevine leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CF pH pH 2 pH 10</td>
<td>Culture filtrate</td>
<td></td>
</tr>
<tr>
<td>Botriosphaeria dothidea</td>
<td>B70</td>
<td>5.85</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>BP04</td>
<td>5.36</td>
<td>0.0</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>B21</td>
<td>5.62</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Diploida mutila</td>
<td>B36</td>
<td>5.53</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>FF18</td>
<td>5.47</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Diploida seriata</td>
<td>A141a</td>
<td>7.15</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>H141a</td>
<td>4.26</td>
<td>0.0</td>
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</tr>
<tr>
<td></td>
<td>ME21b</td>
<td>6.04</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>F321b</td>
<td>7.32</td>
<td>3.0</td>
<td>1.0</td>
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<tr>
<td>Dothiorella vidmadera</td>
<td>L5</td>
<td>5.00</td>
<td>3.0</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>J4</td>
<td>4.74</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>Dothiorella sp.</td>
<td>J24</td>
<td>4.88</td>
<td>2.5</td>
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<tr>
<td>Neofusicoccum australe</td>
<td>VP13</td>
<td>5.84</td>
<td>2.5</td>
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</tr>
<tr>
<td></td>
<td>DNW8</td>
<td>5.24</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>FF10</td>
<td>5.57</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>Neofusicoccum luteum</td>
<td>H12-1</td>
<td>5.91</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>HP19-1</td>
<td>5.96</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>B175</td>
<td>5.94</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Neofusicoccum parvum</td>
<td>E12b</td>
<td>7.96</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>B19a</td>
<td>8.34</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>B22a</td>
<td>7.20</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Spencermartinsia viticola</td>
<td>L19</td>
<td>7.50</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>J7</td>
<td>7.90</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>J8</td>
<td>7.25</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>SDW</td>
<td>–</td>
<td>–</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>SDW (3% MeOH)</td>
<td>–</td>
<td>–</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>25% Czapek-Dox broth</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

aSeverity scale: (0) no symptoms; (1) slight wilting; (2) moderate wilting, necrotic spots; (3) severe necrosis and shrivelling. The experiment were performed in triplicate.

Figure 1. Symptoms caused by 2 mg mL\(^{-1}\) culture filtrate of eight Botryosphaeriaceae isolates on leaves of Vitis vinifera cv. Shiraz in an in vitro bioassay: a) no symptoms (0); b) slight wilting (1); c) moderate wilting, necrotic spots (2); d) severe necrosis and shrivelling.

N. luteum, and S. viticola all produced severe necrosis and shrivelling, while no symptoms were observed in any of the negative controls (Figure 1).

To study the ability to produce lipophilic phytotoxic metabolites, the culture filtrates of all isolates were separated into three aliquots (30 mL each) and their pH modified as follows: unmodified pH, acid pH, and basic pH. In a preliminary experiment, ethyl acetate was shown to be the optimal solvent between those used. The highest yield in organic extract was obtained when the culture filtrates were extracted...
at acid pH (Table S2), which was in the range of 2.0–23.9 mg/30 mL, followed by that obtained from the unmodified pH with yields ranging 1.0–28.5 mg/30 mL, while the lowest was at basic pH with a range of 1.0–14.4 mg/30 mL. The extract residues were assayed on tomato seedlings (Lycopersicon esculentum L.) as reported in the experimental section. The symptoms varied between species, isolates and extraction conditions (Table 1). All the extracts from B. dothidea and N. parvum showed very low phytotoxicity in all the conditions. For D. seriata, D. mutila, D. vidmadera, Dothiorella sp., N. australis, N. luteum and S. viticola the phytotoxicity was strictly dependent on the pH. Considering both the phytotoxic activity and the extract weight, the greatest yield of phytotoxic metabolites for the majority of isolates was obtained extracting under acid conditions. However, similar results for phytotoxicity were obtained for D. mutila (FF18), D. seriata H141a, D. vidmadera L5, N. australis VP13, N. luteum B175 and S. viticola L19.

For all isolates, the residual aqueous phases from the extraction under the unmodified pH condition were assayed on grapevine leaves. Those of N. australis, N. luteum and N. parvum showed phytotoxicity, probably due to the presence of high molecular weight phytotoxins as previously reported by Martos et al. (2008) and Andolfi et al. (2012) but no further investigation was carried out. The residues of the organic extracts were also analyzed by TLC on silica gel and the chromatograms are shown in Figure S2. They showed a relationship between the level of phytotoxicity and the number of secondary metabolites, which all the isolates of B. dothidea and N. parvum revealing a small number of metabolites present in low amounts. D. mutila FF18, showed more metabolites than the strain B36, in particular in the acid extract, and the same was for D. seriata H141a, N. australis VP13, N. luteum B175 and S. viticola L19. The best D. vidmadera metabolite producer was the isolate L5 when its culture filtrates were extracted under unmodified pH conditions. All these results demonstrate a close correlation between the production of lipophilic low molecular weight compounds and phytotoxicity, thus some of these compounds could be defined as phytotoxins.

From the organic extracts of culture filtrates from D. vidmadera L5 were previously isolated five phenols as tyrosol, benzene-1,2,4-triol, resorcin, 3-(hydroxymethyl)phenol and protocatechuic alcohol (1–5, Figure 2) an the new one 5-hydroxymethyl-2-isopropoxyphenol (protocatechuic alcohol isopropyl ether, 6, Figure 1) with 3 having the highest phytotoxicity on grapevine leaves (Vitis vinifera cv. Shiraz) (Reveglia, Baaijens-Billones et al. 2018). From S. viticola L19 were isolated a new phytotoxic dispyridine-butane-1,4-diol and a new diacrylic acid derivatives, named spencertoxin and spencer acid (7 and 8, Figure 2) together with p-hydroxybenzaldehyde and 2-(4-hydroxy-phenyl) acetic acid (9 and 10, Figure 2). 7, 9 and 10 showed phytotoxicity when assayed on grapevine leaves of Vitis lambrusca and V. vinifera cv. Shiraz (Reveglia, Savocchia et al. 2018).

The data reported in Tables 1 and S2 also suggested that further investigations could be conducted to isolate and characterize the phytotoxins present in the organic extract of Diplodia mutila FF18, Diplodia seriata H141a, and Neofusicoccum australis VP13 and Neofusicoccum luteum B175. Thus, a larger cultures were produced and the already known metabolites were identified by comparison of their spectroscopic (essentially 1H and ESI MS) and physic ([α]25D) data with those previously reported.
From culture filtrates of *D. mutila* FF18 were isolated and identified as *R*-(-)-*mellein* (Abou-Mansour et al. 2015) and tyrosol (Kimura and Tamura 1973) (11 and 1, Figure 2). *R*-(-)-*mellein* (11) was also isolated from the culture filtrates of *D. seriata* H141a, *N. australe* VP13, *N. luteum* B175, while tyrosol (1) was also isolated from *N. australe* VP13, *N. luteum* B175. Finally, from this latter were also isolated (3*R*,4*R*)- (Krohn et al. 2007; Abou-Mansour et al. 2015) and (3*R*,4*S*)-4-hydroxy (Devys and Barbier 1992; Abou-Mansour et al. 2015) *melleins* (12 and 13, Figure 2). This is the first reports of fungal phytotoxins isolated from an isolate of *N. luteum*.

Compounds 1 and 11–13 have been already reported as fungal phytotoxins produced by Botryosphaeriaceae species and their phytotoxicity on tomato cuttings and grapevine leaves is well known (Evidente et al. 2010; Abou-Mansour et al. 2015). 11 has also been isolated from the infected grapevine wood (Abou-Mansour et al. 2015). As emphasised in a recent perspective on fungal phytotoxins isolated from grapevine pathogens (Masi et al. 2018), 11–13 are typical phytotoxins produced by Botryosphaeriaceae species involved in grapevine trunk disease. These results further enhanced the idea to use them as biomarker for develop method the early recognize the disease (Masi et al. 2018). However, the role of 11–13 in pathogenicity is still not completely clear and further studies on their mode of action are needed.

Investigation of the others chromatographic fractions obtained from *D. mutila* FF18, *D. seriata* H141a, *N. australe* VP13 and *N. luteum* B175 is ongoing.

In summary, an investigation on the production of secondary metabolites from 24 isolates of eight different Botryosphaeriaceae species associated with Botryosphaeria dieback of grapevines in Australia was conducted. The conditions for the extraction of secondary metabolites from culture filtrates were optimized and the biological assays on host and non-host plants of the culture filtrates and the corresponding organic extracts were performed. The patterns of the secondary metabolites produced by all isolates were determined by chromatographic analysis. The isolation of compounds 1
and 11–13 from *D. seriata*, *D. mutila*, *N. australe* and *N. parvum* raise the question about the absence of foliar symptoms in Australia and their role in Botryosphaeria dieback must by further investigate. To reach this result additional studies will be conducted to isolate and characterize other secondary metabolites produced by isolates of *D. mutila*, *D. seriata*, *N. australe* and *N. luteum* used in this work.

3. Experimental

This section is available as supplementary materials.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References


2.3 SUPPLEMENTARY MATERIAL

2.3.1 Methods

2.3.2 General experimental procedure

Optical rotations were measured in MeOH on a Jasco P-1010 digital polarimeter (Jasco, Tokyo, Japan). $^1$H NMR spectra were recorded at 400 or 500 MHz in CDCl$_3$ on Bruker (Karlsruhe, Germany) and Varian (Palo Alto, CA, USA) instruments. The same solvent was also used as an internal standard. ESI MS and LC/MS analyses were performed using the LC/MS TOF system AGILENT (Agilent Technologies, Milan, Italy) 6230B, HPLC 1260 Infinity. Analytical and preparative TLCs were carried out on silica gel (Kieselgel 60, F254, 0.25 and 0.5 mm respectively) plates (Merck, Darmstadt, Germany). The spots were visualised by exposure to UV radiation, or by spraying first with 10% H$_2$SO$_4$ in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min. Column chromatography was performed using silica gel (Kieselgel 60, 0.063-0.200 mm) (Merck).

2.3.3 Fungal isolates and culture conditions

Twenty-four isolates belonging to nine Botryosphaeriaceae species were studied for their ability to produce phytotoxins in vitro (Table S1). Namely, four isolates of D. seriata, two isolates of D. mutila, three isolates each of B. dothidea, N. australe, N. luteum, N. parvum and S. viticola, two isolates of Do. vidmadera and one Dothiorella sp. were investigated for the production of secondary metabolites. All Botryosphaeriaceae isolates tested in this study (Table S1) were obtained from grapevines showing symptoms of trunk diseases in a SA and NSW vineyards (Pitt et al., 2010; Pitt et al., 2013) and stored at the Australian Scientific Collections Unit (DAR, Orange, NSW, Australia). For toxin production, all isolates were grown individually in flasks containing 100 ml of modified Czapek–Dox liquid medium.
with 0.5% yeast and 0.5% malt extract (pH 6.8) under stationary conditions. Each flask was inoculated with seven mycelial plugs of each isolate previously grown on potato dextrose agar (PDA) for 1 week. The cultures were incubated at 25°C in the dark for 14 days after which the mycelial mats were removed by filtration through four layers of filter paper and kept at -20°C until further processing. A second batch of the liquid culture was prepared as above and incubated for 21 days, filtered and stored at -20°C.

### 2.3.4. Extraction of toxins from fungal culture filtrates

Extractions were carried out at three different pHs: (1) unmodified pH of culture filtrates (pH ranged from 6.6 to 8.7 among the species tested); (2) pH 2, by acidification with 1N formic acid; and (3) pH 10, by alkalinisation with 1N NH₃ according to a procedure previously reported (Martos et al., 2008). For each fungal isolate, three samples of 30 ml were taken from the culture filtrates and their pH modified according to the above procedures. Then each sample was extracted with different organic solvents with increasing polarity: n-hexane, dichloromethane, chloroform and ethyl acetate. The culture filtrates were extracted three times (30 ml each extraction) with each solvent. Organic phases corresponding to the same pH value were then combined, dried over Na₂SO₄, filtered, and evaporated under reduced pressure. The organic residues obtained from each isolate, pH value, and their corresponding aqueous phases, were tested for phytotoxicity as explained below.
2.3.5 General procedure for TLC analysis

Analytical TLC was performed on silica gel (Kieselgel 60, F254, 0.25 and 0.5 mm, respectively) and on reverse phase (Kieselgel 60 RP-18, F254, 0.20 mm) plates (Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation (254 nm and 365 nm), or by spraying first with 10% H₂SO₄ in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110°C for 10 min. Exposure to UV radiation is a method to visualise natural compounds containing a chromophore, the use of two wavelength permit the differentiation between fluorescent and non fluorescent compounds. The spraying with a solution of strong oxidant reagents is the general detector used to reveal compounds belonging to almost all natural substance classes also including those containing chromophores.

2.3.6 Phytotoxicity assays

All culture filtrates and organic extracts obtained from each isolate were tested on grapevine (V. vinifera cv. Shiraz) leaves for their phytotoxicity. For each culture filtrate, a 25% dilution using sterile distilled water (SDW) was prepared and used in the assay. For the dry organic extracts, each were dissolved in 100 μl methanol and the volume adjusted to 3 ml with SDW (2 mg ml-1 solution). Leaves were cut from the 5th youngest node of glasshouse grown grapevines. The petiole of each leaf was immersed in a vial containing 1 ml of the diluted filtrate or 1 ml of organic extract for 20 h inside a growth chamber at 28°C under 12 h light/12 h darkness. Leaves were then transferred to a new vial with 2 ml SDW, and maintained for an additional 28 h period. SDW, SDW with 3% MeOH and 25% Czapek-Dox broth were used as negative controls. Lesions on the leaf surface were evaluated after 28 h after being transferred to SDW using a 0 to 3 scoring scale: 0, no symptoms; 1, slight wilting of
the leaf; 2, moderate wilting of the leaf; 3, severe wilting of the leaf (with occasional necrosis). Each experiment was conducted in triplicate.

The toxicity of the organic extracts was further assayed on 2 week-old tomato seedlings (*Lycopersicon esculentum* L., cv. Grosse Lisse). The compounds were dissolved in 100 μl methanol and the volume adjusted to 3 ml with SDW (2 mg ml⁻¹ solution). Roots were removed and the tomato seedlings immersed in the solution for 24 h. The rootless seedlings were kept in an incubator at 28°C with 12 h light/12 h darkness period during the assay. Seedlings were transferred to SDW under the same light and temperature conditions for further 6 hours. Lesions were evaluated using the same scoring scale reported above. Each experiment was conducted in triplicate.

### 2.3.7 Purification of Low Molecular Weight Phytotoxins from the Organic Extracts of *D. seriata* H141a, *D. mutila* DAR79127, *N. australe* DAR79506 and *N. luteum* DAR81016.

The culture filtrates (4 L) of *D. mutila* DAR79127 was lyophilised and dissolved in 1/10 distilled water of the original volume. The solution was acidified with HCOOH up to pH 2 and extracted with EtOAc (3 x 300 ml). The organic extracts were combined, dried (Na₂SO₄), and evaporated under reduced pressure. The corresponding residue (470 mg) was purified by silica gel column chromatography, eluted with CHCl₃-i-PrOH (95:5, v/v), yielding seven homogeneous fraction groups. The residue of fraction one (10 mg) was purified by TLC eluted with CHCl₃, yielding a white amorphous solid, identified as (R)-mellein (3.6 mg). The residue of fraction five (26.4 mg) was purified on reverse phase TLC, eluted with Me₂CO-H₂O (4:6 v/v), yielding a white solid identified as tyrosol (3 mg). The culture filtrate of *D. seriata* H141a (4 L) was extracted as reported above for *D. mutila* and yielded a brown residue (400 mg), which was purified by silica gel column chromatography
eluted with CHCl₃-i-PrOH (9:1, v/v). Five homogeneous fractions were collected and the residue of fraction one (10.7 mg) was further purified by preparative TLC on silica gel, using n-hexane- EtOAc (6:4, v/v) as an eluent, yielding a white solid which was identified as (R)-mellein (5.2 mg). The culture filtrates (10 L) of *N. austral*e DAR79506 was lyophilised and the corresponding residue re-dissolved in distilled water 1/10 of the original volume. The solution (pH 5) was exhaustively extracted with EtOAc (3 x 500 ml) and the organic extracts were combined, dried (Na₂SO₄), and evaporated under reduced pressure to give a brown oil (1.1 g). This latter was purified by silica gel column chromatography, using CHCl₃-i-PrOH (9:1, v/v) as an eluent, yielding eight homogeneous fraction groups. The residue of fraction two (17 mg) was purified by TLC, eluted with CH₃Cl, yielding a white amorphous solid identified as (R)-mellein (0.6 mg). The residue of fraction four (47 mg) was purified on reverse phase TLC, using MeOH-H₂O (1:1 v/v), yielding tyrosol (1.2 mg). The culture filtrates of *N. luteum* DAR81016 (10 L) were lyophilised and dissolved in 1/10 distilled water of the original volume. The solution was processed as previously described with *D. mutila*. The corresponding residue (511 mg) was purified by silica gel column chromatography, eluted with CHCl₃-i-PrOH (95:5, v/v), yielding eight homogeneous fraction groups. Residue fraction one (8.7 mg) was purified by preparative TLC, eluted with CHCl₃, yielding (R)-mellein (0.6 mg). Residue fraction two (18.1 mg) was purified by preparative TLC, using CHCl₃-MeOH (97:3, v/v) as an eluent, yielding a mixture of two metabolites. This mixture was further purified by TLC on reverse phase, eluted with EtOH-H₂O (1:1, v/v), yielding a white solid, identified as (3R,4S)-(-) and (3R,4R)-(-)-4-hydroxymellein (2 mg and 1.9 mg, respectively). Finally, the residue of fraction five (7.1 mg) was purified by preparative TLC, eluted with n-hexane-Me2CO (1:1 v/v), yielding a tyrosol (2.5 mg). Tyrosol, (R)-Mellein 1, (3R,4R)-(-)-4-Hydroxymellein and (3R,4S)-(-)-4-Hydroxymellein were identified by comparing their spectroscopic data (¹H-NMR and ESI/MS) with those reported in the literature and listed below.
Tyrosol: \( ^1H \) NMR, \( \delta: 7.20 \) (d, \( J = 8.0 \) Hz, H-3 and H-5), 6.80 (d, \( J = 8.0 \) Hz, H-2 and H-6), 4.90 (s, OH), 3.80 (t, \( J = 6.4 \) Hz, H2-1'), 2.80 (t, \( J = 6.4 \) Hz, H2-2'). ESI/MS (+), m/z: 295 \([2 \text{M} + \text{Na}]^+\), 159 \([\text{M} + \text{Na}]^+\). These data are in agreement with Kimura and Tamura 1973.

\((R)\)-Mellein: \([\alpha]_{25}D -93.0 \) (c 2.0); \( ^1H \) NMR (500 MHz), \( \delta: 11.03 \) (s, HO-8), 7.41 (t, \( J = 8.4 \) Hz, H-6), 6.89 (d, \( J = 8.4 \) Hz, H-7), 6.69 (d, \( J = 8.4 \) Hz, H-5), 4.74 (tq, \( J = 6.9 \) and 6.3 Hz, H-3), 2.93 (d, \( J = 6.9 \) Hz, H2-4), 1.53 (d, \( J = 6.3 \) Hz, Me-3). ESI/MS (+), m/z: 179 \([\text{M} + \text{H}]^+\). These data are in agreement with Cole and Cox (1981), Devys et al. (1992), Cabras et al. (2006), Djoukeng et al. (2009), Evidente et al. (2010) and Abou-Mansour et al. (2015).

\((3R,4R)-(−)-4\)-Hydroxymellein: \([\alpha]_{25}D -29.0 \) (c 1.2); \( ^1H \) NMR (400 MHz), \( \delta: 10.99 \) (s, HO-8), 7.55 (t, \( J = 7.6 \) Hz, H-6), 7.03 (d, \( J = 7.6 \) Hz, H-7), 7.00 (d, \( J = 7.6 \) Hz, H-5), 4.70 (dq, \( J = 6.1 \) and 2.1 Hz, H-3), 4.57 (d, \( J = 2.1 \) Hz, H-4), 1.53 (d, \( J = 6.1 \) Hz, Me-C3). ESI/MS (+), m/z: 195 \([\text{M} + \text{H}]^+\). These data are in agreement with Cole and Cox (1981), Krohn et al. (2007), Cabras et al. (2006), Djoukeng et al. (2009), Evidente et al. (2010) and Abou-Mansour et al. (2015).

\((3R,4S)-(−)-4\)-Hydroxymellein: \([\alpha]_{25}D -27.0 \) (c 1.1); \( ^1H \) NMR (400 MHz), \( \delta: 10.99 \) (s, HO-8), 7.55 (t, \( J = 7.6 \) Hz, H-6), 7.03 (d, \( J = 7.6 \) Hz, H-7), 7.00 (d, \( J = 7.6 \) Hz, H-5), 4.60 (m, H-3 and H-4), 1.53 (d, \( J = 6.1 \) Hz, Me-C3). ESI/MS (+), m/z: 195 \([\text{M} + \text{H}]^+\). These data are in agreement with Cole and Cox (1981), Devys et al. (1992), Cabras et al. (2006), Evidente et al. (2010) and Abou-Mansour et al. (2015).
Table S1. Isolates of *Botryosphaeriaceae* species selected for the production and characterisation of secondary metabolites.

<table>
<thead>
<tr>
<th><em>Botryosphaeria</em> spp.</th>
<th>Isolate</th>
<th>aDAR No.</th>
</tr>
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<tr>
<td><em>Botryosphaeria dothidea</em></td>
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<td>DAR79241</td>
</tr>
<tr>
<td></td>
<td>BP04</td>
<td>DAR79242</td>
</tr>
<tr>
<td></td>
<td>B21</td>
<td>-</td>
</tr>
<tr>
<td><em>Diplodia mutila</em></td>
<td>B36</td>
<td>DAR79129</td>
</tr>
<tr>
<td></td>
<td>FF18</td>
<td>DAR79137</td>
</tr>
<tr>
<td><em>Diplodia seriata</em></td>
<td>A142a</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>H141a</td>
<td>-</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>F321b</td>
<td>-</td>
</tr>
<tr>
<td><em>Dothiorella vidmadera</em></td>
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<td>DAR78993</td>
</tr>
<tr>
<td></td>
<td>J4</td>
<td>DAR78992</td>
</tr>
<tr>
<td><em>Dothiorella sp.</em></td>
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<td>DAR78991</td>
</tr>
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</tr>
<tr>
<td></td>
<td>FF10</td>
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</tr>
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</tr>
<tr>
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<tr>
<td></td>
<td>B19a</td>
<td>DAR78998</td>
</tr>
<tr>
<td></td>
<td>B22a</td>
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<td><em>Spencermartinsia viticola</em></td>
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</tr>
<tr>
<td></td>
<td>J7</td>
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<td></td>
<td>J8</td>
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aDAR, Australian Scientific Collections Unit, Orange, NSW Australia.
Table S2. Yields of crude organic extracts obtained from stationary liquid cultures of 24 *Botryosphaeriaceae* isolates associated with Botryosphaeria dieback in Australian vineyard.

<table>
<thead>
<tr>
<th><em>Botryosphaeria</em> spp.</th>
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<th>pH 10 (mg)</th>
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<td></td>
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<td>21.7</td>
<td>7.2</td>
</tr>
<tr>
<td><em>Diplodia seriata</em></td>
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<td>1.9</td>
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<td>2.2</td>
</tr>
<tr>
<td></td>
<td>-</td>
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<td>8.5</td>
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</tr>
<tr>
<td></td>
<td>DAR79236</td>
<td>2</td>
<td>4.6</td>
<td>1.3</td>
</tr>
<tr>
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<td>-</td>
<td>1.3</td>
<td>4.6</td>
<td>0.6</td>
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<td>10.1</td>
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<td></td>
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<td>19.9</td>
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<td><em>Neofusicoccum parvum</em></td>
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<td>1.2</td>
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\(^a\)unmodified pH of culture filtrate
Figure S1. Symptoms of a typical brown wedge-shaped lesion observed in the wood of grapevine infected with *Botryosphaeriaceae* spp. from Australian vineyards.

Figure S2. TLC profiles of *Botryosphaeriaceae* isolates: a) *Dothiorella vidmadera* DAR78993 visualised by spraying with 10% H$_2$SO$_4$ in MeOH and then with 5% phosphomolybdic acid in EtOH, b) *Spencermartinsia viticola* DAR78870 visualised by 254 nm UV light, c) *Diplodia seriata* H141a visualised by 365 nm UV light, d) *Diplodia mutila* DAR79137 visualised by spraying with 10% H$_2$SO$_4$ in MeOH and by 5% phosphomolybdic acid in EtOH, d) *Neofusicoccum australe* DAR79506 visualized by 254 nm UV light; and e) *Neofusicoccum luteum* DAR81016 visualised by 254 nm UV light.
2.4 Research Paper – Spencertoxin and Spencer Acid, New Phytotoxic Derivatives of Diacrylic Acid and Piryridinbutan-1,4-Diol Produced by *Spencermartinsia viticola*, a Causal Agent of Grapevine Botryosphaeria Dieback in Australia.


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**ORIGINAL ARTICLE**

**Spencertoxin and spencer acid, new phytotoxic derivatives of diacrylic acid and dipyridinbutan-1,4-diol produced by *Spencermartinsia viticola*, a causal agent of grapevine Botryosphaeria dieback in Australia**

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**KEYWORDS**  
Grapevine; Botryosphaeria dieback; *Spencermartinsia viticola*; Phytotoxins; Spencertoxin and spencer acid

**Abstract** *Spencermartinsia viticola* is one of the most widespread Botryosphaeriaceae species isolated from grapevines in South Australia and New South Wales vineyards in Australia. A new phytotoxic dipyridine-butane-1,4-diol and a new diacrylic acid derivatives, here named spencertoxin (1) and spencer acid (2), were isolated from the culture filtrates of *S. viticola* isolate DAR78870 together with *p*-hydroxybenzaldehyde (3) and 2-(4-hydroxyphenyl) acetic acid (4). Spencertoxin and spencer acid were characterized as 2,3-di(pyridin-3-yl)butane-1,4-diol and (2<sup>Z</sup>,2<sup>0</sup>Z)-3,3<sup>0</sup>-bis(carboxybis(oxy))diacrylic acid, respectively, by spectroscopic methods (essentially NMR and HRESIMS). Spencertoxin (1), *p*-hydroxybenzaldehyde (3) and 2-(4-hydroxyphenyl) acetic acid (4) showed phytotoxicity when the pure compounds were assayed on grapevine leaves of *Vitis lambrusca* and *Vitis vinifera* cv. Shiraz.

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Peer review under responsibility of King Saud University.

**1. Introduction**

Botryosphaeria dieback, caused by fungi belonging to the family Botryosphaeriaceae, is one of the major trunk diseases affecting grapevines. Over the past few decades, the incidence of symptoms of trunk diseases has increased considerably worldwide. Prevention of Botryosphaeria dieback currently
rely on modifying the timing of grapevine pruning practices and protection of pruning wounds, while management of the disease relies on remedial surgery (Ayres et al., 2016; Ayres et al., 2017; Savocchia et al., 2017). Due to the lack of synthetic fungicides registered for the control of Botryosphaeria dieback an increasing number of chemists and plant pathologists are studying various aspects of this disease. Symptoms of the disease in infected vines include characteristic wedge-shaped wood necrosis of the trunk and cordsons. Moreover, foliar symptoms associated with the disease have also been reported (Uirbez-Torres, 2011). Leaf spots are associated with the production of phytotoxins by the pathogen which are translocated to the leaves from the wood (Tabacchi et al., 2000). Apart from the stunting of shoots, to date no foliar symptoms have been detected in Australian vineyards affected by Botryosphaeria dieback (Pitt et al., 2013a). The absence of leaf spots raises questions about the capability of Botryosphaeriaceae isolated from grapevines in Australia to produce phytotoxins.

Different phytotoxins are produced by fungi involved in grapevine trunk diseases and these have been chemically characterised and tested for their toxicity on the leaves of various Vitis species and on non-host plants (Andolfi et al., 2011). These phytotoxins belong to different classes of organic compounds such as naphthalenone pentaketides like isoclerone and scytalone produced by fungi involved in the Esca complex. Diplodia seriata and Neofuscosicicum parvum, two of the most widespread and virulent pathogens, are known to produce phytotoxic metabolites belonging to the isocoumarin family called melleins (Martos et al., 2008; Andolfi et al., 2011). Neofuscosicum australiae haplotype H4, associated with grapevine cordon dieback, produced structurally different secondary metabolites such as a new phytotoxic cyclohexenone oxide, named cyclobotryoxide, together with 3-methylcatechol and tyrosol (Andolfi et al., 2012) in vitro. Lasiodiplodia species were also investigated for their production of phytotoxic metabolites. L. mediterranea, recently isolated from grapevine and closely related to L. pseudotoxobaeria, produces in vitro three jasmonic acid esters, named lasiojasmonates A-C, and 16-O-acetylbortyosphaerillactones A and C, (1R,2R)-jasmonic acid, its methyl ester, bortyosphaerillactone A, (3S,4R,5R)-4-hydroxymethyl-3,5-dimethyl-2-furanone, and (3R,4S)-botryodiploidin (Andolfi et al., 2014). In a recent study, phytotoxic metabolites produced in liquid culture by six species of Lasiodiplodia isolated in Brazil and causing Botryosphaeria dieback of grapevine, were chemically identified. As ascertained by LC-MS, L. brasiliense, L. crassipora, L. jatrophiolica, L. pseudotoxobaeriae produced jasmonic acid, and L. brasiliense, synthesized jasmonic acid and (3R,4S)-4-hydroxyxymethyl. L. euphorbiola produced (−)-mellein, (3R,4R)-(−) and (3R,4S)-(−)-4-hydroxymellein and tyrosol, and L. hormozganensis synthesized tyrosol and p-hydroxybenzoic acid (Cimmino et al., 2017). Furthermore, fungi involved in Botryosphaeria dieback produced phytotoxic polyaccharides (EPSs), some of which were also recently chemically and biologically characterized (Cimmino et al., 2016).

A study on the identification, prevalence and distribution of Botryosphaeriaceae species in vineyards in winegrowing regions of eastern Australia resulted in eight species from four phylogenetic lineages being isolated from grapevines (Pitt et al., 2010). These species included D. seriata, Diplodia mutila, L. theobromae, N. parvum, N. australiae, Botryosphaeria dothidea, Dothiorella viticola (syn. Spencermartinsia viticola) and Dothiorella vidmadera. Neofuscosicicum luteum and Neofuscosicicum ribis were also isolated in a separate survey of vineyards in eastern Australia (Savocchia et al., 2007; Wunderlich et al., 2011; Pitt et al., 2013b).

This manuscript reports the isolation and chemical and biological characterization of new phytotoxic dipyridine-butane-1,4-diol and diacrylic acid derivatives, named spencertoxin and spencer acid, isolated from the culture filtrates of S. viticola (DAR78870) together with, p-hydroxybenzaldehyde and p-hydroxyphenyl acetic acid.

2. Materials and methods

2.1. General experimental procedure

IR spectra were recorded as a deposit glass film on a Thermo Electron Corporation Nicolet 5700 FT-IR spectrometer (Madison, WI, USA) and UV spectra were measured in MeCN on a Jasco V-530 spectrophotometer; 1H and 13C NMR spectra were recorded at 400 or 500 and 100 or 125 MHz in CDCl3 on Bruker (Karlsruhe, Germany) and Varian (Palo Alto, CA, USA) instruments. The same solvent was used as an internal standard. The multiplicity were determined by DEPT spectra (Berger and Braun, 2004). The same solvent was also used as an internal standard. DEPT, COSY-45, HSQC and HMBC were performed using Bruker and Varian microprograms (Berger and Braun, 2004). HRESIMS and LC/MS analyses were performed using the LC/MS TOF system (AGILENT 6230B, HPLC 1260 Infinity, Milan, Italy, column Phenomenex LUNA (C18) (2) 5 μ 150 × 4.6 mm). Analytical and preparative TLCs were carried out on silica gel (Kieselgel 60, F254, 0.25 and 0.5 mm respectively) and on reverse phase (Kieselgel 60 RP-18, F254, 0.20 mm plates) (Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation, or by first spraying with 10% H2SO4 in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed using silica gel (Kieselgel 60, 0.063–0.200 mm) (Merck).

2.2. Fungal strains and culture conditions

The isolate of S. viticola (DAR78870) used in this study was obtained from a grapevine showing symptoms of trunk diseases in a South Australia vineyard (Pitt et al., 2010) and was stored at the Australian Scientific Collections Unit (Orange, NSW, Australia). The isolate was grown in stationary conditions in four flasks containing 2 L of modified Czapek–Dox medium with 0.5% yeast and 0.5% malt extract (pH 6.8). Each flask containing the medium was inoculated with 15 mycelium plugs of 1-week-old S. viticola cultured on potato dextrose agar. The cultures were incubated at 25 °C in the dark for 14 h, after which the mycelial mats were removed by filtration through four layers of filter paper and kept at −20 °C until further processing.

2.3. Extraction and purification of phytotoxins

The lyophilized residues of the culture filtrates of S. viticola (8 L) were dissolved in 500 ml of distilled water. This latter was...
extracted with EtOAc (3 × 60 ml) at pH 2. The organic extracts were combined, dried (Na₂SO₄), filtered and evaporated under low pressure, yielding a brown oily residue (1.12 g). This residue was purified by silica gel column chromatography, eluted with CHCl₃:MeOH (95:5) as the eluent and yielded a yellow homogeneous solid which was identified as p-hydroxybenzaldehyde (3) (Fig. 1, 1.3 mg, 0.16 mg/L, Rₚ 0.5) as reported below. The residue (80.8 mg) of fraction three, purified on preparative TLC on silica gel, using CHCl₃:MeOH (90:8:2), yielding two amorphous solids, here named spencertoxin and spencer acid (1 and 2, Fig. 1, 4.7 mg, 0.2 mg/L, Rₚ 0.2, and 1.4 mg, 0.18 mg/L, Rₚ 0.4, respectively).

2.4. Identification of compound 1-4

Compounds 3 and 4 were identified by comparing their spectroscopic data (¹H NMR and ESI/MS) with those already reported in the literature for p-hydroxybenzaldehyde (3) (Andolfi et al., 2015) and 2-(4-hydroxyphenyl)acetic acid (4) (Zhang et al., 2011).

Spencertoxin (1). UV: λₘₐₓ: nm (log e) 261 (3.1); IR: νₘₐₓ: 3372, 2969, 2915, 2850, 1694, 1645, 1384 cm⁻¹; ¹H and ¹³C NMR: Table 1; HRESIMS (+) m/z: 267.1100 [M + Na]⁺ (calcd. for C₁₄H₁₆N₂NaO₂ 267.1109, [M+Na]+). Spencertoxin (1) ¹H and ¹³C NMR data of spencertoxin (1).a,b

<table>
<thead>
<tr>
<th>Position</th>
<th>δ C</th>
<th>δ H (J in Hz)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4</td>
<td>70.7t</td>
<td>3.60 (2H) dd (11.2, 4.8)</td>
<td>H-2,3</td>
</tr>
<tr>
<td>2,3</td>
<td>62.9d</td>
<td>3.65 (2H) m</td>
<td>H-1,4</td>
</tr>
<tr>
<td>2,2''</td>
<td>150.1d</td>
<td>9.13 (2H) br s</td>
<td></td>
</tr>
<tr>
<td>3,3''</td>
<td>148.1s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4,4''</td>
<td>137.9d</td>
<td>8.40 (2H) br d (7.7)</td>
<td></td>
</tr>
<tr>
<td>5,5''</td>
<td>123.7d</td>
<td>7.55 (2H) br v (7.7)</td>
<td></td>
</tr>
<tr>
<td>6,6''</td>
<td>151.5d</td>
<td>8.69 (2H) br s</td>
<td></td>
</tr>
</tbody>
</table>

* The chemical shifts are in δ values (ppm) from TMS.

Spencer acid (2). UV: λₘₐₓ: nm (log e) 357 (2.9), 258 (3.7); IR: νₘₐₓ: 3347, 1721, 1664 cm⁻¹; ¹H and ¹³C NMR: Table 2; HRESIMS (+) m/z: 225.0019 [M + Na]⁺ (calcd. for C₇H₆O₇ Na₂O 225.0017).

<table>
<thead>
<tr>
<th>Position</th>
<th>δ C</th>
<th>δ H (J in Hz)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>165.9s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,2''</td>
<td>142.1d</td>
<td>7.39 (2H) d (7.7)</td>
<td>H-3,3'</td>
</tr>
<tr>
<td>3,3''</td>
<td>102.5d</td>
<td>5.62 (2H) d (7.7)</td>
<td>H-2,2'</td>
</tr>
<tr>
<td>4,4''</td>
<td>174.9s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The chemical shifts are in δ values (ppm) from TMS.

2.5. Phytotoxicity bioassays

The phytotoxic activity of crude extract chromatographic fractions was assayed on non-host lemon fruits. The samples were dissolved in dimethylsulfoxide (DMSO) and diluted in distilled water, up to a final concentration of 1 mg/ml and 4% DMSO. The lemon fruit surface was sterilized with sodium hypochlorite (50 µg/ml) and, subsequently, washed with three rinses of sterile distilled water (SDW). The fruit surface was treated with a 10 µl droplet of the test solution and the treated area wounded three times using a sterile needle. SDW and 4% DMSO solution were used as negative controls. The treated fruit was maintained at room temperature (16–22 °C) and visually assessed after 72 h for necrotic spots. Compounds 1-4 were tested on grapevine leaves (Vitis lambrusca and Vitis vinifera cv. Shiraz). The compounds were dissolved in 100 µl methanol and the volume adjusted to 3 ml with distilled water (100 µg/ml, 10⁻³ M solution). Grapevine leaves were harvested from glasshouse grown grapevines and the petioles immersed in 1 ml of the phytotoxic solutions. SDW and 4% DMSO solution were used as negative controls. The petiole of each leaf was immersed in a vial containing 1 ml of the filtrate dilution for 20 h. Leaves were then transferred to a new vial with 2 ml SDW, placed in a growth chamber with 12 h light / 12 h darkness period at 28 °C and maintained for an additional 28 h period. Lesions on the leaf surface were evaluated using a 0–3 scale: 0, no symptoms; 1, slight wilting of the leaf; 2, moderate wilting of the leaf; 3, severe wilting of the leaf (with occasional necrosis). Each treatment was conducted in triplicate.
3. Results and discussion

The organic extract obtained from the culture filtrates of *S. viticola* was purified following a bioguided fractionation and two new metabolites were isolated together with two already known ones (Fig. 2). The new metabolites were named spencertoxin and spencer acid (1 and 2, Fig. 1), and the other two were identified as *p*-hydroxybenzaldehyde and 2-(4-hydroxyphenyl)acetic acid (3 and 4, Fig. 1). Compounds 3 and 4 are already known metabolites and were identified by comparing their spectroscopic (1H NMR and HRESIMS) properties with those reported in earlier studies (Andolfi et al. 2015; Zhang et al. 2011).

Spencertoxin (1) had a molecular formula C_{14}H_{16}N_{2}O_{2} as deduced from its HRESIMS and consistent with eight hydrogen deficiencies. Its 1H and COSY (Berger and Braun, 2004) spectra (Table 1) showed a broad singlet, a broad triplet (J = 7.7 Hz), a broad doublet (J = 7.7 Hz) and a broad singlet at δ 8.69 (H-60), 7.55 (50), 8.40 (40) and 9.13 (H-20) typical signal system of a 3-monosubstituted pyridine ring. A to d doublets (J = 11.2 and 4.8 and J = 11.2 and 5.8 Hz) due to the protons of a hydroxylated methylene (H2-1) was observed at δ 3.60 and 3.52, as also coupled with benzylic methine proton (H-2) appearing as multiplet at δ 3.65 (Pretsch et al., 2000). These systems and the molecular weight suggest a dimeric structure for 1. Thus, the assignment of the proton is the same for those of the second half of the compound (Table 1). These signals were also in full agreement with the band for hydroxyl and aromatic groups observed in the IR spectrum (Nakanishi and Solomons 1977) as well as the absorption maximum recorded in the UV spectrum (Pretsch et al., 2000). The couplings observed in the HSQC spectrum (Table 2) (Berger and Braun, 2004) allowed the assigning of the carbons resonating in the 13C NMR spectrum (Table 1) at δ 151.5, 150.1, 137.9, 123.7, 70.7 and 62.9 to the protonated carbons C-6,60, C-2,20, C-4,40, C-5,50, C-1,4 and C-2,3. Thus the residual signal at δ 148.1 was assigned to the two quaternary carbons to C-3,30 (Table 1).

On the basis of these findings spencertoxin (1) was formulated as 2,3-di(pyridin-3-yl)butane-1,4-diol. This structure was confirmed by the long-range couplings observed in the HMBC spectrum (Table 1) (Berger and Braun, 2004) and by the data of its HRESIMS spectrum. This latter spectrum showed the sodiated cluster [M + Na]+ at m/z 267.1100 and a significant ion at m/z 123 corresponding to the protonated half molecule [M/2 + H]+.

Pyridine derivatives are well known as naturally occurring compounds such as nicotinic acid, which is formed in humans from the metabolism of dietary tryptophan involving the intermediate kynurenine. The substance is a precursor of the nicotinamide adenine dinucleotide and related metabolites and alkaloid present in foodstuff and tobacco products (Zwickenpflug et al., 2016). Fusaric acid is a well-known mycotoxin produced by several different *Fusarium* species reported as pathogens of cereals (Bacon et al., 1996). Furthermore, *Fusarium nygamai* which produces fusaric acid, was proposed for the biocontrol of *Striga hermonitica* (Capasso et al., 1996), a devastating hemiparasitic plant. Several different *Fusarium* species have been proposed for the biocontrol of *Orobanche ramosa*, an holoparasitic plant responsible for major losses to vegetable, legume, and sunflower crops by interfering with water and mineral intake and by affecting photosynthate partitioning (Abouzeid et al., 2004).

Spencer acid (2) had a molecular formula of C_{7}H_{6}O_{7} as deduced from its HRESIMS spectrum consistent with five hydrogen deficiencies. The 1H NMR and COSY spectra
Spencertoxin and spencer acid

(Table 2) showed two doublets \((J = 7.7 \text{ Hz})\) of a cis-disubstituted double bond at \(\delta 7.39\) and 5.62 (Pretsch et al., 2000). The \(^{13}\text{C}\) NMR spectrum as well as the HSQC spectra (Table 2) showed the corresponding olefinic carbons at \(\delta \) 142.1 and 102.5 in accordance with the oxygenate nature of C-2. Furthermore, the signals of two carboxylic carbonyls were observed at \(\delta\) 174.9 and 165.9, respectively (Breitmaier and Voelter, 1987). These signals agreed with the bands for hydroxyl and carbonyls groups present in the IR spectrum (Nakanishi and Solomons, 1977) and the maxima recorded in the UV spectra (Pretsch et al., 2000).

Considering these finding and the molecular formula, 2 appeared to be a symmetrical compound and in particular a diacylic acid derivative. Furthermore, the couplings observed in the HMBC spectrum (Table 1) between the signal at \(\delta\) 165.9 with H-2,3 and H-3,3 allowed the identified the carbon 1 as the anhydride carbonyl, while the residual signal at \(\delta\) 174.9 was assigned to the carbons of carboxylic acid groups.

On the basis of these findings spencer acid could be formulated as \((2Z,2Z)-3,3'-(\text{carboxylbis(oxy)})\text{diacrylic acid}\) (2).

The structure assigned to 2 was supported from the other couplings observed in the HMBC spectrum (Table 1) and from its HRESIMS. The last spectrum showed the sodium cluster at \([\text{M + Na}^+]/m/z\) 225.0019.

The isolation of low molecular weight mono- and di-carboxylic acids as phytotoxins has already been reported from bacteria and fungi. Phenylacetic acid was isolated from Biscogniauxia mediterranea involved in the cork oak canker disease (Evidente et al., 2005), nitropropanoic acid was isolated from Septoria cirsii and Melanconis thelebola, and more recently from Diaporthe gulyae. The latter three fungi were proposed as mycoherbicides for the control of Canada thistle (Hershernhorn et al., 1993), red alder (Alies rubra Bong), a forest infesting species (Evidente et al., 1992) and Carthamus lana- tus, a widespread winter-growing annual weed of both pastures and crops throughout Australia (Andolfi et al., 2015). p-Hydroxy- and p-methyl benzoic acid has been also isolated from D. gulyae, and when grown in a bioreactor, succinic acid was isolated as the main metabolite (Andolfi et al., 2015). 3-acetoxy-25-methylopropanoic, fumaric acid, p-hydroxybenzoic acid, vanillic acid, (-)-sydonic acid, furan-2-carboxylic acid were produced from Epichloe bromicola, a fungus isolated from Wildrye grass (Elymus tangerorum) and some of these showed antifungal activity (Song et al., 2015).

Compounds 1-4, were assayed on grapevine leaves at concentrations of 100 \(\mu\)g/ml \((10^{-3}\text{ M})\). The most phytotoxic compounds on grapevine leaves were \(p\)-hydroxybenzaldehyde (3) and 2-(4-hydroxyphenyl)acetic acid (4) followed by spencer-toxin (1), while spencer acid (2) produced no symptoms of phytotoxicity under the test conditions (Table 3).

Considering the appearance of necrosis and wilting on grapevine leaves when 1, 3 and 4 were assayed in vitro (Fig. 3) and the absence of foliar symptoms on naturally infected grapevine in Australia, further investigations are required to clarify the role of phytotoxic metabolites in the pathogenicity and symptom expression of Botryosphaeria dieback pathogens in Australian vineyards.

### Table 3 Level of toxicity induced 28 h after treatment on Vitis lambrusca and Vitis vinifera cv. Shiraz leaves by compounds produced by Spencermartinsia viticola.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Level of toxicity</th>
<th>Vitis lambrusca</th>
<th>Vitis vinifera (cv. shiraz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spencertoxin (1)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Spencer acid (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(p)-hydroxybenzaldehyde (3)</td>
<td>2.5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2-(4-hydroxyphenyl)acetic acid (4)</td>
<td>3</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>SDW</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SDW 3% MeOH</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Severity scale: (0) no symptoms; (1) slight wilting; (2) moderate wilting, necrotic spots; (3) severe necrosis and shrivelling.

### Fig. 3 Symptoms caused by compounds 1 and 3 on leaves of Vitis vinifera cv. Shiraz, after in vitro bio-assaying at \(10^{-3}\text{ M}\): a severe necrosis and shrivelling caused by 3; b moderate wilting and necrotic spots caused by 1; c symptomless leaf (negative control SDW).

4. Conclusion

To our knowledge this is the first report of the isolation of secondary metabolites from S. viticola, a pathogen involved in grapevine trunk diseases. A new dipyridine-butane-1,4-diol and a new diacylic acid derivatives, named spencertoxin and spencer acid, were isolated from the culture filtrates of S. viticola together with, \(p\)-hydroxybenzaldehyde and 2-(4-hydroxyphenyl) acetic acid. The phytotoxicity of S. viticola was also discussed in relation to the symptoms caused in vivo on grapevine leaves.

This achievement provides new knowledge on the ability of S. viticola to produce phytotoxins in vitro however further investigations are required to clarify the role of these phytotoxins in the disease cycle of Botryosphaeria dieback.
Acknowledgements

Antonio Evidente is associated with “Istituto di Chimica Biomolecolare del CNR”.

Contributors

Pierluigi Reveglia, Sandra Savocchia, Regina Billones-Baaijens, Marco Masi and Antonio Evidente designed the study, analyzed the data and wrote the manuscript. Pierluigi Reveglia performed the experiments.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.arabjc.2018.01.014.

References


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Isolation of Phytotoxic Phenols and Characterization of a New 5-Hydroxymethyl-2-isopropoxyphenol from Dothiorella vidmadera, a Causal Agent of Grapevine Trunk Disease

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ABSTRACT: Polyphenols were characterized from Dothiorella vidmadera (DAR78993), which was isolated from a grapevine in Australia. In total, six polyphenols were isolated including a new polyphenol characterized by a spectroscopic method (essentially NMR and HR ESIMS) as 5-hydroxymethyl-2-isopropoxyphenol. Tyrosol, benzene-1,2,4-triol, resorcinol, 3-(hydroxymethyl)-phenol, and protocatechuic alcohol, the latter being the main metabolite, were also isolated. Although these are already known as naturally occurring compounds in microorganisms and plants, this is the first time they have been isolated from fungal organisms involved in grapevine trunk disease. When assayed on tomato seedlings, all the compounds show similar phytotoxic effects. However, when assayed on grapevine leaves (Vitis vinifera cv Shiraz), resorcinol was the most toxic compound, followed by protocatechuic alcohol and 5-hydroxymethyl-2-isopropoxyphenol.

Keywords: grapevine, Botryosphaeria dieback, Dothiorella vidmadera, phytotoxins, protocatechuic alcohol isopropyl ether

INTRODUCTION

Different phytotoxins are produced by plant pathogenic fungi involved in grapevine trunk diseases, and these have been previously chemically characterized and tested for their toxicity on the leaves of various Vitis species and on nonhost plants. These phytotoxins belong to different classes of organic compounds. Pathogenic fungi belonging to the family Botryosphaeriaceae are involved in various diseases that affect grapevines worldwide. One of the most important diseases caused by this family of fungi is Botryosphaeria dieback, and over the past few decades, the incidence of disease symptoms has increased, causing economic and yield losses worldwide. Currently, no curative methods are available for this disease and only methods to prevent the disease are available. The disease symptoms include dieback of the wood, cankers, and a characteristic wedge-shaped wood lesion of the trunk and cordon. Furthermore, foliar symptoms associated with the disease have also been reported. At least 27 Botryosphaeriaceae species have been isolated from grapevines and all are implicated in Botryosphaeria dieback. To date, 10 Botryosphaeriaceae species have been isolated from vineyards in the wine-growing regions of eastern Australia, and these include Diplodia seriata, Diplodia mutila, Lasiodiplodia theobromae, Neofusicoccum parvum, Neofusicoccum australe, Botryosphaeria dothidea, Dothiorella viticola (syn. Spencermartinsia viticola), Dothiorella vidmadera, Neofusicoccum luteum, and Neofusicoccum ribis.

Several phytotoxins have been isolated and characterized from a number of Botryosphaeriaceae species. Melleins, phytotoxic metabolites belonging to the isocoumarin family, are known to be produced by D. seriata and N. parvum, two of the most widespread and virulent pathogens. Neofusicoccum australe haplotype H4, associated with grapevine cordon dieback, produced structurally different secondary metabolites in vitro, such as a new phytotoxic cyclohexenone oxide named cyclobotryoxide, together with 3-methylcatechol and tyrosol. In a recent study, phytotoxic metabolites produced in liquid culture by six species of Lasiodiplodia isolated from infected grapevine wood in Brazil and causing Botryosphaeria dieback were chemically identified. As ascertained by LC-MS, L. brassiciflora, L. crassigera, L. stapfiana, and L. pseudoeuphorbiae produced jasmonic acid, and L. brassiciflora synthesized jasmonic acid and (3R,4S)-4-hydroxymellein. Lasiodiplodia euphorbicola produced (−)-mellein, (3R,4R)-(-)-, and (3R,4S)-(−)-4-hydroxymellein and tyrosol, while L. hormozganensis synthesized tyrosol and p-hydroxybenzoic acid. While the role of the phytotoxins in pathogenicity and symptomology is not completely clear, an interesting hypothesis could be that they are involved in the expression of foliar symptoms and after production by the pathogen the phytotoxins are translocated to the leaves from the wood. So far, no foliar symptoms have been detected in Australian vineyards affected by Botryosphaeria dieback. The absence of these symptoms raises questions about the capability of Botryosphaeriaceae
isolated from grapevines in Australia to produce phytotoxins. *Dothiorella vidmadera* is usually classified as a weak pathogen; however, it is one of the most widespread Botryosphaeriaceae species in South Australian vineyards.12 To date, there is no information reported on the phytotoxic metabolites produced by *D. vidmadera* in liquid culture.

This manuscript reports the isolation and chemical and biological characterization of a new phytotoxic 5-hydroxymethyl-2-isopropoxyphenol, isolated from the culture filtrates of *D. vidmadera* (DAR78993)3 together with benzene-1,2,4-triol, resorcinol, 3-(hydroxymethyl)phenol, protocatechue acid, and tyrosol.

**MATERIALS AND METHODS**

**General Experimental Procedure.** IR spectra were recorded as a deposit glass film on a Thermo Electron Corporation Nicolet 5700 FT-IR spectrometer (Madison, WI, USA), and UV spectra were measured in MeCN on a Jasco (Tokyo, Japan) V-530 spectrophotometer; 1H and 13C NMR spectra were recorded at 400 or 500 and 100 and 125 MHz, respectively, in CDCl3, unless otherwise noted, on Bruker (Karlsruhe, Germany) and Varian (Palo Alto, CA, USA) instruments. The same solvent was used as an internal standard. The multiplicities were determined by DEPT spectra.1 The same solvent was also used as an internal standard. DEPT, COSY-45, HSQC and HMBC were performed using Bruker and Varian microprograms.13 HR ESI/MS and LC/MS analyses were performed using the LC/MS TOF system (AGILENT 6230B, HPLC 1260 Infinity, Milan, Italy) column Phenomenex LUNA (Torrance, CA, USA) (C18 (2) 50x150 nm). Analytical and preparative TLCs were carried out on silica gel (Kieselgel 60, 0.25 and 0.5 mm respectively) and on reverse phase (Kieselgel 60 RP-18, F254, 0.20 mm) plates (Merck, Darmstadt, Germany). The spots were visualized by exposure to UV radiation, or by spraying first with 10% H2SO4 in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed using silica gel (Kieselgel 60, 0.063–0.200 mm) (Merck).

**Fungal Strains and Culture Conditions.** The isolate of *D. vidmadera* (DAR78993) used in this study was obtained from a grapevine showing symptoms of trunk diseases in a South Australian vineyard and was stored at the Australian Scientific Collections Unit (Orange, NSW, Australia).11,12 The isolate was grown under stationary conditions in four flasks containing 2 L of modified Czapek–Dox medium with 0.5% yeast and 0.5% malt extract (pH 6.8). Each culture containing the medium was inoculated with 15 mycelial plugs of the isolate grown on potato dextrose agar (PDA) for 1 week. The cultures were incubated at 25 °C in the dark for 14 days after which the mycelial mats were removed by filtration through four layers of filter paper and kept at −20 °C until further processing.

**Extraction and Purification of Phytotoxins.** The lyophilized residues of the culture filtrates of *D. vidmadera* were dissolved in 500 mL of distilled water. The organic phase was extracted with EtOAc (3 × 600 mL) at pH 5.7. The organic extracts were combined, dried (Na2SO4), filtered, and evaporated under reduced pressure, yielding a new compound of an amorphous solid identified as 3-(hydroxymethyl)phenol (1), which was further purified by preparative TLC, using CHCl3-MeOH-AcOH (9:8:0.2, v/v) as an eluent, resulting in a white homogeneous solid which was identified as resorcinol (3), 3-(hydroxymethyl)phenol (4), protocatechue alcohol (5), tyrosol (6), and triacetyl protocatechuic alcohol (7).

![Figure 1. Structures of protocatechue alcohol isopropyl ether (1), benzene-1,2,4-triol (2), resorcinol (3), 3-(hydroxymethyl)phenol (4), protocatechue alcohol (5), tyrosol (6), and triacetyl protocatechuic alcohol (7).](image)
Table 1. $^1$H and $^{13}$C NMR Data of 5-Hydroxymethyl-2-isopropoxyphenol (1)$^{a,b}$

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$^a$The chemical shifts are in δ values (ppm) from TMS. $^b$2D $^1$H,$^1$H (COSY) and 2D $^{13}$C,$^1$H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons. Multiplicities were assigned by DEPT spectra.

RESULTS AND DISCUSSION

Chromatographic column fractions of purified organic extracts obtained from the culture filtrates of D.vidmadera were initially tested for their phytotoxicity on lemon fruits. One new metabolite, named 5-hydroxymethyl-2-isopropoxyphenol (1, Figure 1), was isolated together with benzene-1,2,4-triol, resorcinol, 3-hydroxymethyphenol, protocatechuic alcohol and tyrosol (2–6, Figure 1). Compounds 2–6 have already been reported as fungal and plant metabolites and were identified by comparing their spectroscopic ($^1$H NMR and ESI/MS) properties with those reported in the literature. $^{14,17}$

5-Hydroxymethyl-2-isopropoxyphenol (1) had a molecular formula of C$_{10}$H$_{14}$O$_3$ as deduced from its HRESIMS and was consistent with four hydrogen deficiencies. Its $^1$H and COSY spectra (Table 1) showed a broad singlet and a two broad doublets ($J = 8.0$ Hz) resonated at $\delta$ 6.52 (H-6), 6.66 (H-3), and 6.75 (H-4), as expected for the signals system of a trisubstituted benzene ring, while a singlet, due to the protons of a hydroxylated methylene (H$_2$-7) was observed at $\delta$ 4.64. Similarly, a quartet ($J = 6.1$ Hz) and a doublet ($J = 6.1$ Hz), typical signals of an isopropyl group, resonated at $\delta$ 3.75 (H-8) at 1.25 (H-9 and 10$^\prime$) and two broad singlets due to two hydroxyl groups were recorded at $\delta$ 7.46 (OH-C-2) and 4.35 (HO-C-7).$^{19}$ These signals were also in full agreement with the hydroxyl and aromatic bands observed in the IR spectrum$^{19}$ as well as with the absorption maximum recorded in the UV spectrum.$^{16}$ The couplings observed in the HSQC spectrum (Table 1) allowed the assignment of the carbons resonating in the $^{13}$C NMR spectrum (Table 1) at $\delta$ 137.3, 120.7, 117.2, 115.7, 114.6, 72.0, 69.5, and 22.9 due to the protonated carbons C-3, C-4, C-6, C-7, C-8, and C-9/C-10. The two tertiary sp$^3$ carbons observed at $\delta$ 145.1 and 146.1 were assigned to C-1 and C-2 for their couplings observed in the HMBC spectrum (Table 1) with HO-C-1, H-3, H-6, and HO-C-1, H-3, H-4, and H-6, respectively. Finally, the quaternary sp$^2$ carbon observed at $\delta$ 137.3 was assigned to C-5 for the long-range coupling observed in the same spectrum with H-6 and H-7.$^{20}$

The structure of 1 was confirmed by the data of its HR ESIMS spectrum which showed a potassium cluster [M + K]$^+$ at m/z 221.0019.

5-Hydroxymethyl-2-isopropoxyphenol (1) and metabolites 2–6 are polyphenols and thus belong to the group of polyketides that also include a diverse group of natural products. The function of the extracts is unknown; however, it is believed that they function as pigments, as virulence factors, as info-chemicals, or for defense.$^{21}$ To our knowledge this is the first time that compounds 1–5 have been isolated and characterized from a fungal organism isolated from a grapevine with symptoms of trunk disease.

Benzen-1,2,4-triol (2) is already known as a fungal and plant secondary metabolite. 2 has been isolated from fungi belonging to species of Aspergillus$^{22}$ from Gardenia jasminoides fruits$^{23}$ and from the leaves of Cinnamoum parthenoxylon (Jack).$^{24}$

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Resorcinol (3) is another well-known phytotoxic metabolite isolated from medicinal plants, and it was also found as a decomposed product of corn and rye residues in the soil. Several secondary metabolites with diverse biological activity isolated from fungi and bacteria possess a structure directly related to a resorcinol moiety. 3-(Hydroxymethyl)phenol (4) is a secondary metabolite usually associated with antioxidant and free radical scavenging activity. 4 has been isolated from fungi, including different species of Penicillium and from the secretions of Nicrophorus vespilloides. Proto-catechuic alcohol (5) was the main phytotoxic compound isolated from D. vidmadera and has been already reported as a fungal metabolite produced by a mangrove fungus BYY-1. The same authors also assayed the antitumor activity of 5, showing that it significantly inhibits the proliferation of Hela cells. Tyrosol (6) is a well-known phytotoxic metabolite produced by plants and by several fungi, including N. parvum and D. seriata. This phytotoxic metabolite was more recently isolated from different strains of Lasiodiplodia involved in grapevine trunk disease in Brazil. Their phytotoxicity on grapevine leaves and tomato seedlings has already been reported. Compounds 1–5 and 7 were assayed on grapevine leaves (V. vinifera cv Shiraz) and tomato seedlings at concentrations of 100 μL (10⁻³ M) as described above (Table 2). The most phytotoxic compound on grapevine leaves was resorcinol (3), causing severe shrivelling of the leaves while protocatechuic alcohol (5) and 5-hydroxymethy-2-isopropoxyphenol (1) caused moderate shrivelling of the leaves (Figure 2). 3-(Hydroxymethyl)phenol (4) and 1,2,4-O,O′,O″-triacetyl protocatechuic alcohol (7) caused only slight wilting (Figure 2). 1,2,4-Benzotetrol (2) did not show any phytotoxic symptoms on grapevine leaves under the test conditions. Conversely, when assayed on tomato seedlings, compounds 1–5 and 7 showed essentially the same level of phytotoxicity. These results suggest that the grapevine leaves may be less susceptible to the phytotoxic metabolites assayed because they may be structurally similar to polyphenols which are involved in the plants’ defense against trunk disease pathogens.

This study provides new knowledge on the ability of D. vidmadera to produce phytotoxins in vitro. Considering the absence of foliar symptoms in Australian vineyards, further investigations are required to clarify the role of phytotoxic metabolites in the pathogenicity and symptom expression of Botryosphaeria dieback pathogens in grapevines.

### Table 2. Level of Toxicity Induced 28 h after Treatment on Tomato Seedlings cv. Grouse and Vitis vinifera cv. Shiraz Leaves by Metabolites (1–5 and 7) Produced by Dothiorella vidmadera

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<td>7</td>
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</table>

Severity scale: (0) no symptoms; (1) slight wilting; (2) moderate wilting, necrotic spots; (3) severe necrosis and shrivelling.

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**Figure 2. Symptoms caused by compounds 1, 3, and 4 on leaves of Vitis vinifera cv. Shiraz, in vitro at 10^-3 M of 1, 3, and 4:** (a) severe necrosis and shrivelling caused by 3; (b) moderate wilting and necrotic spots caused by 1; (c) slight wilting caused by 4; (d) symptomless leaf (negative control SDW).

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**REFERENCES**


2.6 Research Paper – Diploquines A and B, Two New Phytotoxic Tetrasubstituted 1,4-Naphtoquines from Diplodia mutila, a Causal Agent of Grapevine Trunk Disease.


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Diploquinones A and B, Two New Phytotoxic Tetrasubstituted 1,4-Naphthoquinones from Diplodia mutila, a Causal Agent of Grapevine Trunk Disease

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† Dipartimento di Scienze Chimiche, Università di Napoli Federico II, Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Napoli, Italy
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ABSTRACT: Two new phytotoxic tetrasubstituted 1,4-naphthoquinones, named diploquinones A and B, were isolated together with vanillic acid from Diplodia mutila (DAR78993), a grapevine pathogen involved in Botryosphaeria dieback in Australia. Diploquinones A and B were characterized as 6,7-dihydroxy-2-methoxy-5-methylnaphthalene-1,4-dione and 3,5,7-trihydroxy-2-methoxynaphthalene-1,4-dione using spectroscopic methods (essentially 1D and 2D 1H and 13C NMR and HR ESIMS). The already known vanillic acid was isolated for the first time as fungal phytotoxic and as metabolite of D. mutila. The three compounds were assayed on detached grapevine leaves (Vitis vinifera cv. Shiraz) at concentrations of 10−3 M and 2.5 × 10−3 M. Vanillic acid showed the highest phytotoxic effect on grapevine leaves irrespective of the tested concentration, while diploquinones A and B showed varying degrees of toxicity.

KEYWORDS: grapevine, Botryosphaeria dieback, Diplodia mutila, phytotoxins, diploquinones A and B

Introduction

Botryosphaeriaceae species are known to cause disease in several important agricultural crops, forest trees, and ornamental plants. In the past decade, the importance of diseases caused by these species on grapevines has increased significantly. Among them, Botryosphaeria dieback is one of the most significant grapevine trunk diseases threatening the sustainability of vineyards worldwide and at least 26 different species in the Botryosphaeriaceae have been associated with Botryosphaeria dieback. Currently, no curative methods are available for grapevine trunk diseases which cause significant losses in yield.

The characteristic symptoms of Botryosphaeria dieback include leaf spots, shoot and branch dieback, bud necrosis, cankers, and necrotic wedge shaped lesions in the trunks and cordon of infected vines. Some of these symptoms, and in particular the leaf spots, have been associated with the production of phytotoxic secondary metabolites by the fungi involved in grapevine trunk diseases. Many efforts have been made to determine the structure and mode of action of these phytotoxic metabolites; however, further studies are required to determine their role in the pathogenicity and to understand their relationship with the observed symptoms.

Botryosphaeria dieback is also an important disease in Australia, and considerable efforts have been made to understand the causal organisms and their management. At least 11 Botryosphaeriaceae species have been isolated from infected grapevines in Australian vineyards; however, no foliar symptoms have been observed to date. Limited information is available on the capability of these pathogens to produce phytotoxic metabolites in Australian vineyards. Thus, a preliminary screening was conducted to determine if eight different species of Botryosphaeriaceae isolated from grapevines in Australia could produce phytotoxic secondary metabolites. The species involved in this investigation were Botryosphaeria dothidea, Diplodia seriata, Diplodia mutila, Dothiorella vidmadera, Neofusicoccum australe, Neofusicoccum luteum, Neofusicoccum parvum, and Spencermartinsia viticola. Among them, D. seriata, D. mutila, D. vidmadera, N. australe, N. luteum, and S. viticola produced the greatest amount of organic extracts and were the most phytotoxic when bioassayed on grapevine leaves. Phytotoxins from D. vidmadera and S. viticola were recently characterized and identified in vitro. D. vidmadera produced six polyphenols, one of them isolated and characterized for the first time, while S. viticola produced a derivative of diacrylic acid and a dipyrindibutan-1,4-diol. The toxicity of these compounds was tested on both grapevine leaves and tomato seedlings in vivo. Preliminary analysis of an organic extract from D. mutila resulted in the characterization of R-(−)-mellein and tyrosol, two previously reported phytotoxic secondary metabolites.

This manuscript further reports the production of phytotoxic secondary metabolites from D. mutila and, in particular, the isolation and chemical and biological characterization of two new phytotoxic tetrasubstituted 1,4-naphthoqui-
nones, named diploquinones A and B, together with vanillic acid.

**MATERIALS AND METHODS**

**General Experimental Procedures.** These procedures were previously reported.10

**Fungal Strains and Culture Conditions.** The isolate of *D. mutila* (DAR79137) used in this study was obtained and grown as previously reported.10,13

**Extraction and Purification of Secondary Metabolites.** The culture filtrates (6 L) of *D. mutila* (DAR79137) were lyophilized and dissolved in 1/10 distilled water of original volume. The solution was acidified with HCOOH at pH 2 and exhaustively extracted with EtOAc (3 × 300 mL). The organic extracts were combined, dried (Na2SO4), and evaporated under reduced pressure. The corresponding residue (470 mg) was purified by silica gel column chromatography and eluted with CHCl3/i-PrOH (95/5, v/v), yielding seven homogeneous fraction groups. The residue of fraction one (10 mg) was purified by TLC eluted with CHCl3, resulting in an orange amorphous solid, characterized, as below reported, and named diploquinone A (1, 7 mg). The residue of fraction two (26.4 mg) was purified on silica TLC, eluted with CHCl3/i-PrOH (98/2, v/v), yielding a yellow amorphous solid characterized, as below reported, and named diploquinone B (2, 2 mg). The residue of fraction four (15 mg) was further purified by preparative TLC on silica gel, using EtOAc/i-PrOH (95/5, v/v) as an eluent, affording a white solid which was identified as 4-hydroxy-3-methoxybenzoic acid (3, 3.3 mg), also known as vanillic acid.

**Diploquinone A (1).** IR (KBr) ν max 3675, 3594, 1772, 1541 cm⁻¹; UV λ max (log ε) 255 (4.20), 330 (3.40); 1H- and 13C NMR data see Table 1; HRESI MS (+), m/z 257.0433 (C12H10NaO5, calcld 257.0426, [M + Na]+).

**Diploquinone B (2).** IR (KBr) ν max 3590, 3457, 1771, 1650, 1570 cm⁻¹; UV λ max (log ε) 287 (4.10), 327 (3.40), 450 (3.10); 1H- and 13C NMR data see Table 1. ESI MS (+), m/z 259.0213 (C11H9NaO5, calcld 259.0219 [M + Na]+).

**Vanillic Acid (3).** 1H NMR δ 7.71 (d, J = 8.2, 1.8 Hz, H-6), 7.59 (d, J = 1.8 Hz, H-2), 6.97 (d, J = 8.2 Hz, H-5); ESI MS (+), m/z 167 [M – H]⁻. These data are in agreement with those previously reported.14

**Methyl Ester of Vanillic Acid (4).** An ethereal solution of diazomethane was added to 1 mg (0.05 mmol) of vanillic acid (3) dissolved in MeOH (0.5 mL). The reaction was stirred for 1 h, and then the mixture was evaporated under reduced pressure. The crude residue (1.5 mg) was purified by preparative TLC using n-hexane/Me2CO (7/3, v/v) as the eluent, yielding the corresponding derivative 4 (0.8 mg). The 1H NMR differed from 3 by the presence of a methoxy group at δ 3.89. ESI MS (+) m/z: 205 [M + Na]².

**Phytotoxic Bioassay.** Compounds 1–3 were dissolved in 40 μL of MeOH, and the volume was adjusted to 1 mL in sterile distilled water (SDW). The bioassays were conducted at two different concentrations, 10⁻³ M and 2.5 × 10⁻³ M. Disease-free grapevine leaves were harvested from the fifth node of glasshouse grown Vitis vinifera cv. Shiraz, and the petiole of each leaf was immersed in a vial containing 1 mL of each of the solutions for 20 h. SDW with 4% MeOH were used as negative controls. The leaves were then transferred to a new vial with 2 mL SDW, placed in a growth chamber with 12 h light/12 h darkness period at 28 °C and maintained for an additional 28 h period. Lesions on the leaf surface were visually recorded after 48 h. Each experiment was conducted in triplicate.

**RESULTS AND DISCUSSION**

The purified acid organic extract of *D. mutila* resulted in three amorphous solids that were orange, yellow, and white. Following preliminary 1H NMR investigation, the yellow and orange compounds appeared to belong to the 1,4-naphthoquinone class of natural compounds with a different functional group on both A and B rings, while the white solid had a 1,3,4-trisubstituted aromatic structure. For both the orange and yellow metabolites no matches for 1H NMR spectra were found in the literature, and after an extensive 1D and 2D 1H and 13C NMR analysis (reported below) they were characterized as two new 1,4-naphthoquinones and named diploquinones A and B, respectively (1 and 2, Figure 1).

Diploquinone A (1) had a C12H10O5 molecular formula as deduced from its HRESI MS spectrum that was consistent with eight hydrogen deficiencies. An investigation of its 1H NMR spectrum (Table 1) showed the presence of four singlets. The first two were at δ 6.26 and 5.95 (H-8 and H-3), typical of a methoxy group linked to the double bound to the ring B at δ 3.89. ESI MS (+) m/z: 205 [M + Na]². These data are in agreement with those previously reported.15

### Table 1. 1H and 13C NMR Data of Diploquinones A and B (1 and 2)²,⁶

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<td></td>
<td>4a</td>
<td>127.1 C</td>
<td>H-6, H-8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>129.6 C</td>
<td>Me-C(5)</td>
<td></td>
<td>5</td>
<td>153.6 C</td>
<td>HO-5</td>
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<tr>
<td>6</td>
<td>154.8 C</td>
<td>H-8</td>
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<td>6</td>
<td>101.3 CH</td>
<td>6.56 (1H) s</td>
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<td>7</td>
<td>159.5 C</td>
<td></td>
<td></td>
<td>7</td>
<td>155.7 C</td>
<td>H-8</td>
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<td>8</td>
<td>122.3 CH</td>
<td>6.26 (1H) s</td>
<td></td>
<td>8</td>
<td>108.4 CH</td>
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<tr>
<td>8a</td>
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<td>Me-C(5)</td>
<td></td>
<td>8a</td>
<td>139.8 C</td>
<td>H-8</td>
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</tr>
<tr>
<td>OMe-C(2)</td>
<td>56.5</td>
<td>3.87 (3H) s</td>
<td>OMe-C(2)</td>
<td>57.6</td>
<td>CH3</td>
<td>3.92 (3H), s</td>
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<tr>
<td>Me-C(5)</td>
<td>13.7</td>
<td>2.35 (3H) s</td>
<td></td>
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spectrum (Table 1) allowed the assignment of the chemical shifts to the protonated carbons of the 13C NMR spectrum (Table 1) at δ 122.3, 106.8, 56.5, and 13.7 to C-8, C-3, MeO-C(2), and Me-C(5), respectively. The couplings observed in the HMBC spectrum (Table 1) between C-1 and H-8, C-4 and H-3, C-2 and OMe, C-7 and H-8, and C-5 and C-6 both with Me-C(5) allowed the assignment of the chemical shifts to the carbonyls and quaternary aromatic and olefinic sp2 carbons and, namely, the signals at δ 183.7, 178.3, 159.7, 159.5, 154.8, and 129.6 to C-1, C-4, C-2, C-7, C-6 and C-5, respectively. The remaining two signals at δ 125.0 and 121.0 (exchangeable) were assigned to C-4a and C-8a.

Thus, 1 was formulated as 6,7-dihydroxy-2-methoxy-5-methylnaphthalene-1,4-dione. The structure assigned to 1 was supported also by the significant sodium cluster [M + Na]+ recorded in its HR ESIMS spectrum at m/z 257.0433.

Diploquinone B (2, Figure 1) had a molecular formula of C11H8O6 as deduced by its HRESI MS spectrum and was consistent with eight hydrogen deficiencies. Its preliminary 1H and 13C NMR (Table 1) compared with those of 1 differed only for the functionalities of A and B rings. These results are consistent with the bands observed in the IR spectrum for hydroxyl, carbonyl, and aromatic groups and with the maxima absorption exhibited in the UV spectrum. The detailed investigation of the 1H NMR spectrum of 2 showed the presence of two singlets of meta coupled (J < 1 Hz) of two aromatic protons, resonating at the typical chemical shift values of δ 7.59 and 6.56 (H-8 and H-6) and the singlet of the OMe group at δ 3.92. Furthermore, a significant singlet due to a phenolic hydroxy group (HO-5) linked to the adjacent carbonyl group O==C(4) at δ 10.89 was observed. The couplings observed in HSQC spectrum (Table 1) allowed the assignment of the chemical shifts to the protonated sp2 carbon and, namely, those at δ 108.4 and 101.3 to C-8 and C-6, respectively, and the signal of the OMe group resonated at δ 57.6. The long-range couplings observed in the HMBC spectrum (Table 1) among C-2 and HO-3, C-3 and OMe, C-4 and H-8, C-4a and H-6 and H-8, both C-5 and C-6 both with HO-5, and both C-7 and C8a with H-8 allowed the assignment of the signals at δ 154.4, 159.9, 172.0, 127.1 153.6, 155.7, and 139.8 to C-2, C-3, C-4, C-4a, C-5, C-7, and C-8a. The remaining signal observed at δ 181.8 was assigned to the other carbonyl group O==C(1).

Thus, the chemical shifts to all the protons and the corresponding carbons were assigned and reported in Table 1, and 2 was formulated as 3,5,7-trihydroxy-2-methoxynaphthalene-1,4-dione. The structure assigned to 2 was supported also by the significant sodium cluster [M + Na]+ recorded in its HRESI MS spectrum at m/z 259.0213.

Diploquinone B (2) appeared to be a structural isomer of the 1,4-naphthoquinone isolated from Cercospora melonis during a screening of secondary metabolites. No biological activity was reported for the structural isomer of 2.

The white solid was identified as 4-hydroxy-3-methoxybenzoic acid, also known as vanillic acid (3, Figure 1), by comparison of its 1H NMR data with those previously reported. Its ESIMS spectrum showed the pseudomolecular ion [M − H]− at m/z 167.

Figure 1. Structure of diploquinones A and B, vanillic acid, and its methyl ester (1–4).

Figure 2. Symptoms caused by compounds 1–3 on leaves of Vitis vinifera cv. Shiraz in vitro at 10−3 M and 2.5 × 10−3 M: (a) vanillic acid at 10−3 M; (b) vanillic acid at 2.5 × 10−3 M; (c) diploquinone A at 10−3 M; (d) diploquinone A at 2.5 × 10−3 M; (e) diploquinone B at 10−3 M; (f) diploquinone B at 2.5 × 10−3 M; and (g and f) negative control of sterile distilled water with 4% MeOH.
The identification of vanillic acid was confirmed by converting 3 into the corresponding methyl ester (4, Figure 1) by its reaction with an ethereal solution of diazomethane. The $^1H$ NMR spectrum of 4 differed from that of 3 essentially for the presence of the singlet due to the ester methoxyl group at $\delta$ 3.89. Its ESI MS spectrum also showed the pseudomolecular ion [M + Na]$^+$ at m/z 205. These data were very similar to those previously reported when 4 was prepared as a synthetic precursor of bosutinib, an anticancer drug, starting from 3-methoxy-4-hydroxybenzoic acid.\(^{15}\)

Compounds 1–3 were assayed on detached grapevine leaves (Vitis vinifera cv. Shiraz) at concentrations of 10$^{-3}$ M and 2.5 $\times$ 10$^{-3}$ M.

Vanillic acid (3) appeared to be the most toxic compound causing severe shriveling and distortion of the leaf lamina together with necrotic spots in the bioassay for phytotoxicity. The toxic effect of vanillic acid was independent of the two tested concentrations (Figure 2a,b). Diploquinone A tested at 10$^{-3}$ M caused necrotic spots, moderate wilting, and distortion of the leaf lamina. The same compound at 2.5 $\times$ 10$^{-3}$ M concentration induced extensive necrosis in the central part of the leaf lamina and wilting (Figure 2c,d). For diploquinone B assayed at 10$^{-3}$ M, only necrotic spots localized close to the petiole were observed. However, distortion and shriveling of the leaf lamina with necrotic spots were observed at 2.5 $\times$ 10$^{-3}$ M (Figure 2e,f). No phytotoxic symptoms were observed in the leaves that received the negative control treatment (Figure 2g,h).

The present study provides new insight on the ability of D. mutila to produce phytotoxic secondary metabolites. Two new tetrasubstituted 1,4-naphthoquinones, named diploquinones A and B, and vanillic acid were isolated. These results, together with those already reported in the literature,\(^{16}\) broadens the knowledge on phytotoxic metabolites produced by Botryosphaeriaeae species involved in grapevine trunk disease. D. mutila also a canker pathogen of forest plants such as Italian cypress (Cupressus sempervirens L.) and oak trees (Q. ilex, Q. cerri, and Q. robur) is known to produce sphaeropsidins B and C, two phytotoxins belonging to the class of pimarane diterpene.\(^{20}\) On the other hand, a tetrahydropyran-pyran-2-one named diplopyrone was isolated from the oak tree pathogen.\(^{21}\) These results confirm that the same fungal pathogen isolated from different hosts and/or from different environments can produce different secondary metabolites that may play an important role in plant–pathogen interactions. Thus, the comparison of metabolic patterns may assist in future classification/distinction of strains that infect different hosts.

Diploquinones A (1) and B (2) belong to a well-known class of natural compounds of 1,4-naphthoquinones isolated from bacteria, plants, and fungi. Phytotoxic naphthoquinones as well as the related naphthalenones were produced by fungal pathogens of legumes and grapevine; among them are 2- and 3-hydroxyjuglone, botrytone, regiolone, cis- and trans-2,4,8,3-trihydroxynaphthalenones, isolated from B. fabae\(^{22}\) and scytalone, regiolone, cis-4-hydroxyiscytalone, 1,3,8-trihydroxynaphthalene, 3,4,8-trihydroxytetralone, 2,4,8-trihydroxytetralone, and flavolin isolated from Phaeocoreonium minimum, Phaeomoniella chlamydospora, and N. parvum.\(^{17}\) These compounds display many different activities including anticancer, anti-inflammatory, antibiotic, and phytotoxic activities.\(^{23,24}\) Several studies suggest that the structure of naphthoquinone is potentially capable of redox conversions; thus, their antibiotic and phytotoxic effects are due to the interaction with the oxidative systems of microbial and plant cells. Furthermore, this ability depends on the chemical features of the structure of the naphthoquinone.\(^{25}\) The different phytotoxic effects showed by diploquinone A and B could be related to their different substitutions of the rings A and B. The presence of an ortho-diphenols moiety on ring A of diploquinone A makes it more oxidizable, and therefore this could enhance its interaction with the oxidative systems of plant cells. The naphthoquinones can have an opposite role when produced by fungi or by plants. For instance, pathogenic fungi may produce naphthoquinones to inhibit the generation of reactive oxygen species (ROS) in the plant tissue they infect, in order to protect themselves from the defense reaction produced by the plant. Quinone/hydroquinone structures serve as cofactors in many metabolic pathways, playing critical chemical roles in oxidation/reduction processes. This mode of action could also operate for the phytotoxic activity of foeniculoxin, isolated by some of the authors as the phytotoxic geranylhydroquinone produced by Phomopsis foeniculi, the causal agent of fennel stem necrosis.\(^{26}\) Similarly, sphaeropsidones which are fungal cyclohexene oxides isolated from Sphaeropsis sapinea are able to induce the development of haustorium in radicles of the parasitic weeds Striga and Orobanche.\(^{27}\) Naphthoquinones are not the only class of natural compounds that could have this role; even anthraquinones can have this role.\(^{28}\)

Conversely, another study showed that juglone and plumbagin, derivatives of 1,4-naphthoquinone occurring in plants, are able to generate reactive oxygen species (ROS), which play an important role in the processes of programmed cell death during plant defense.\(^{29}\) Hence, naphthoquinones play a key role in plant–pathogen interactions, and future in planta studies may assist in understanding their complex activity.

Vanillic acid (3) belonging to the polyphenols is a well-known plant metabolite that arises from the shikimic and acetic acid (polyketide) metabolic pathways.\(^{30}\) In addition, compound 3, together with other phenolic acids such as gallic, caffeic, and syringic acids, are considered polyphenol allelochemicals. They have been observed in both natural and managed ecosystems where they cause a number of ecological and economic problems.\(^{31}\) These compounds are also well-known for their pharmacological properties and in particular for their anti-inflammatory and antioxidant actives.\(^{32}\) For its biotechnological value vanillic acid was also obtained from microbiological conversion of eugenol, a well-known plant monoterpane.\(^{33}\)

This is the first report of vanillic acid as a fungal phytotoxin and as metabolite of D. mutila and its high phytotoxicity in the bioassay is consistent with previous results where it showed strong toxic effects and growth inhibition on crops such as Pisum sativum and sorghum. Its toxic effect is probably due to a negative influence of the metabolic processes of the mitochondria in plant cells.\(^{34–36}\) The isolation of vanillic acid as a phytotoxin produced by D. mutila is not surprising as it has been recently isolated from D. vidnadera.\(^{1}\) Future studies could focus on understanding if polyphenols secreted by these pathogens may also be involved in the onset of disease symptoms observed in the field.
The authors declare no competing financial interest.

**REFERENCES**


Chapter 3 – Comparative analysis of phytotoxins production by *Neofusicoccum* species involved in Botryosphaeria dieback in Australia

3.1 Introduction
Among all the species of *Botryosphaeriaceae* involved in BD, *Neofusicoccum* spp. are considered highly virulent pathogens and they have been reported in almost all the winegrowing regions (Úrbez-Torres 2011). To date, eight different *Neofusicoccum* spp. have been isolated from infected vines showing BD symptoms in the vineyard (Mondello et al. 2018). However, only four species have been isolated from infected grapevines in Australian vineyards: *N. australae*, *N. luteum*, *N. parvum* and *N. ribis* (Billones-Baaijens et al. 2019). *N. parvum*, together with *D. seriata*, are the most studied species that are associated with grapevine trunk diseases and multidisciplinary approaches have been used to investigate their virulence factors (Úrbez-Torres 2011, Ramírez-Suero et al. 2014, Abou-Mansour et al. 2015, Morales-Cruz et al. 2015, Bénard-Gellon et al. 2015, Stempien et al. 2018). Generally, pathogenic fungi have an array of PMs, that probably act in synergy, that are mediators of virulence and depending on their mode of action, they may interact with different cellular targets, inhibit the activity of plant enzymes or interfere with the biosynthesis of plant hormones (Möbius and Hertweck 2009). It is important to highlight that the mode of action of many PMs are still unknown and further studies are needed. Indeed, it is known that *N. parvum* can produce a plethora of low molecular weight compounds that are toxic on grapevine leaves, tomato leaves and grapevine calli (Evidente et al. 2010, Abou-Mansour et al. 2015). Recently, exopolysaccharides were obtained from the culture filtrates of an *N. parvum* isolate and tested on grapevine leaves showing different degrees of toxicity (Cimmino et al. 2016). However, more in-depth investigations are required to understand the role of these extracellular compounds in the development and expression of disease symptoms. Investigations by Bénard-Gellon et al. (2015) showed that *N. parvum* produced aggressive extracellular proteins, resulting in necrosis and the induction of an up-regulation of grapevine defense genes. Another study further showed that secreted *Botryosphaeriaceae* proteins triggered a high accumulation of δ-viniferin in *V. vinifera* suspension cells, although not sufficient to completely inhibit fungal colonisation (Stempien et al. 2018).
Considerably less information is available on PMs isolated and characterised from *N. australe*. Different PKs along with other known secondary metabolites were characterised from one isolate of *N. australe* obtained from the plant epidermis of *Sonneratia apetala* (a type of mangrove) (Xu et al. 2011). Two different isolates of *N. australe* belonging to two different haplotypes, H4 and H1, were studied by Andolfi et al. (2012). The isolates belonging to haplotype H4 and associated with BD, produced a new cyclohexenone oxide, namely, cyclobotryoxide, 3-methylcatechol and tyrosol. On the other hand, the haplotype H1, associated with branch dieback of *Phoenicean juniper*, produced botryosphaerone D and (3S,4S)-3,4,8-trihydroxy-6-methoxy-3,4dihydro-1(2H)-naphthalenone. Cyclobotryoxide was the most phytotoxic compound (Andolfi et al. 2012).

*Neofusicoccum luteum* is known to be capable of producing phytotoxic extracellular compounds when grown *in vitro* (Martos et al. 2008). However, prior to the investigation reported in Chapter 2, no characterised PMs were available in the literature for *N. luteum* (Reveglia et al. 2018c). (R)-mellein, tyrosol and (3R,4R)-and (3R,4S)-4-hydroxy melleins were isolated and recognized from the organic extract of the culture filtrates of *N. luteum* DAR81016 (Reveglia et al. 2018a). From the same study, the first chemical and biological analyses on culture filtrates and organic extracts of Australian isolates of *N. australe*, *N. luteum* and *N. parvum* was reported. The culture filtrates showed high phytotoxicity when assayed on grapevine leaves but the toxicity of the organic extracts differed among species and isolates (Reveglia et al. 2018a). However, from the bioguided chromatographic purification of their organic extracts, other chromatographic fractions retain the phytotoxic activity, but the very low amount of organic material did not permit any further analysis.

Since significant variation in the virulence between species and isolates was observed, it is reasonable that some variation in the extracellular compounds could occur among species of *Neofusicoccum* involved in BD in Australia. The aim of this work was to evaluate the differences in the production and toxicity of extracellular PMs and EPSs for three of the *Neofusicoccum* spp. involved in BD in Australia.
3.2 Materials and Methods

3.2.1 General Experimental Procedure
IR spectra were recorded as a deposit glass film on a Thermo Electron Corporation Nicolet 5700 FT-IR spectrometer (Madison, WI, USA) and UV spectra were measured in MeCN on a Jasco (Tokyo, Japan) V-530 spectrophotometer; $^1$H and $^{13}$C NMR spectra were recorded at 400 or 500 and 100 or 125 MHz, respectively, in CDCl$_3$, unless otherwise noted, on Bruker (Karlsruhe, Germany) and Varian (Palo Alto, CA, USA) instruments. The same solvent was used as an internal standard. The multiplicity was determined by DEPT spectra (Berger and Braun, 2004). The same solvent was also used as an internal standard. DEPT, COSY-45, HSQC and HMBC were performed using Bruker and Varian microprograms. HR ESIMS and LC/MS analyses were performed using the LC/MS TOF system (AGILENT 6230B, HPLC 1260 Infinity, Milan, Italy) column Phenomenex LUNA (C18 (2) 5 $\mu$m 150x 4.6 mm). Analytical and preparative TLCs were carried out on silica gel (Kieselgel 60, F$_{254}$, 0.25 and 0.5 mm respectively) and on reverse phase (Kieselgel 60 RP-18, F$_{254}$, 0.20 mm) plates. The spots were visualised by exposure to UV radiation or by spraying first with 10% H$_2$SO$_4$ in MeOH, and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 $^\circ$C for 10 min on a hot plate. Column chromatography was performed using silica gel (Kieselgel 60, 0.063-0.200 mm).

3.2.2 Fungal Strains and Culture Conditions
The isolates of *N. australe* DAR79506, *N. luteum* DAR81016 and *N. parvum* DAR80004 used in this study were obtained from the culture collection at the National Wine and Grape Industry (NWGIC), Charles Sturt University (CSU), Australia. These isolates were obtained from grapevine showing symptoms of trunk diseases in Australian vineyards and previously identified by partial DNA sequencing of their ribosomal DNA (Pitt et al, 2010; Qiu et al. 2011; Wunderlich et al. 2011). The isolates were grown under stationary conditions in four flasks containing 2 L of modified Czapek Dox medium with 0.5% yeast and 0.5% malt extract (pH 6.8). Each flask containing 1 L of the medium was inoculated with 15 mycelial plugs of each isolate grown on PDA for 1 week. The 10 L liquid cultures for each isolate were incubated at 25$^\circ$C in the dark for 14 days after which the mycelial mats were removed by filtration through four layers of filter paper and kept at -20$^\circ$C until further processing.
3.2.3 Extraction and Purification of Secondary Metabolites Produced by *N. australe* DAR79506

The culture filtrates (10 L) of *N. australe* DAR79506 were lyophilised and the corresponding residue re-dissolved in distilled water 1/10 of the original volume. The solution (pH 5) was exhaustively extracted with EtOAc (3 x 500 ml) and the organic extracts were combined, dried (Na$_2$SO$_4$), and evaporated under reduced pressure to give a brown oil (1.1 g). This latter was purified by silica gel column chromatography, using CHCl$_3$-i-PrOH (9:1, v/v) as an eluent, yielding eight homogeneous fraction groups. The residue of fraction two (17 mg) was purified by TLC, eluted with CH$_3$Cl, afforded a white amorphous solid identified as (R)-mellein (1, Figure 3.1, 10.6 mg). The residue of fraction three (9 mg) was purified on reverse phase TLC, using MeOH-H$_2$O (1:1 v/v), yielding another amorphous solid identified as tyrosol (2, Figure 3.1, 1.2 mg). The residue of fraction four (38 mg) was purified by TLC, eluted with CH$_3$Cl-MeOH (95:5, v/v), yielding a red amorphous solid characterised as a new anthraquinone and named neoanthraquinone (3, Figure 3.1, 11.8 mg) The $^1$H-NMR and $^{13}$C NMR data are reported in Table 3.1. The residue of fraction five (29 mg) was purified on TLC on silica gel, using EtOAc-MeOH-H$_2$O (85:10:5, v/v), yielding a homogeneous compound identified as p-cresol (4, Figure 3.1, 1.4 mg).

3.2.4 Extraction and Purification of Secondary Metabolites Produced by *N. luteum* DAR81016

The culture filtrates of *N. luteum* DAR81016 (10 L) were lyophilised and dissolved in 1/10 distilled water of original volume. The solution was acidified with HCOOH to achieve pH 2 and exhaustively extracted with EtOAc (3 x 300 ml). The organic extracts were combined, dried (Na$_2$SO$_4$), and evaporated under reduced pressure. The corresponding residue (511 mg) was purified by silica gel column, eluted with CHCl$_3$-i-PrOH (95:5, v/v), yielding eight homogeneous fraction groups. Residue fraction one (9 mg) was purified by preparative TLC, eluted with CHCl$_3$, resulting in (R)-mellein (1, Figure 3.1, 1.6 mg). Residue fraction two (23.1 mg) was purified by preparative TLC, using CHCl$_3$-MeOH (97:3, v/v) as an eluent, resulting in a mixture of two metabolites. This mixture was further purified by reverse phase TLC, eluted in EtOH-H$_2$O (1:1, v/v), yielding a white solid, identified as (3R,4S)- and (3R,4R)-(-)-4-hydroxymellein (5, 6, Figure 3.1, 1.4 mg and 3.2 mg, respectively). Residue fraction three (33 mg) was purified by preparative TLC, using CHCl$_3$-MeOH (97:3, v/v) as an eluent, yielding neoanthraquinone (3, Figure 3.1, 3.7 mg) and another metabolite. The latter was further purified by TLC on silica gel, eluted with CHCl$_3$-iPro (9:1, v/v), yielding an amorphous solid, identified as nigrosporione (7, Figure 3.1, 0.4 mg). The residue of fraction five (7.1 mg) was purified by preparative TLC, eluted with n-hexane-Me$_2$CO (1:1 v/v), resulting in tyrosol (2, Figure 3.1, 2.5 mg).
3.2.5 Extraction and Purification of Secondary Metabolites Produced by *N. parvum* DAR 80004

The culture filtrates of *N. parvum* DAR 80004 (10 L) were lyophilised and dissolved in 1/10 distilled water of original volume. The solution was acidified with HCOOH to achieve pH 2 and exhaustively extracted with EtOAc (3 x 300 ml). The organic extracts were combined, dried (Na$_2$SO$_4$), and evaporated under reduced pressure. The corresponding residue (357.5 mg) was purified by silica gel column, eluted with CHCl$_3$-i-PrOH (95:5, v/v), yielding seven homogeneous fraction groups. Residue fraction one (12.8 mg) was purified by preparative TLC, eluted with CHCl$_3$, resulting in (R)-mellein (1, Figure 3.1, 2 mg) and another two amorphous solids further purified on reverse phase, EtOH-H$_2$O (1:1, v/v), and identified as (3R,4S)- and (3R,4R)(-)4-hydroxymellein (5, 6, Figure 3.1, 1.3 mg and 2.1 mg, respectively). Residue fraction five (8.8 mg) was purified by preparative TLC, eluted with CHCl$_3$-MeOH (95:5, v/v), yielding tyrosol (2, Figure 3.1, 8.3 mg).

3.2.6 Identification of Isolated Compounds

Compounds 1−7 were identified by comparing their spectroscopic data (1H NMR and ESI/MS) with those already reported in the literature and as reported below.

(R)-mellein (1): [α]$^\text{D}$, NMR (400 MHz) and ESI/MS (+) data are in agreement with those previously reported (Djoukeng et al. 2009, Evidente et al. 2010, Abou-Mansour et al. 2015).

tyrosol (2): NMR (400 MHz) and ESI/MS (+) data are in agreement with those previously reported (Kimura and Tamura 1973, Capasso, et al. 1992, Cimmino et al. 2017)

Neoanthraquinone (3): IR $\nu_{\text{max}}$: 3626, 3334, 3274, 2365, 1630, 1555, 1395, 1210. UV $\lambda_{\text{max}}$ nm (log ε) 257 (4.13), 272 (3.45), 327 (2.20); $^1$H NMR (400 MHz),13C-NMR (100MHz) data reported in Table 3.1. ESI/MS (+), m/z: 319.0946 [M + Na]$^+$, 297.1127 [M + H]$^+$.

p-cresol (4): $^1$H-NMR (400 MHz) and ESI/MS (+) data are in agreement with those previously reported (Kasthuraiah et al. 2004, Passmore et al. 2018 ).
(3R,4S)-4-hydroxy mellein (5): \([\alpha]^{25}_D\), \(^1\)H-NMR (400 MHz) and ESI/MS (+) data are in agreement with the data previously reported (Djoukeng et al. 2009, Evidente et al. 2010, Abou-Mansour et al. 2015).

(3R,4R)-4-hydroxy mellein (6): \([\alpha]^{25}_D\), \(^1\)H-NMR (400 MHz) and ESI/MS (+) data are in agreement with the data previously reported (Djoukeng et al. 2009, Evidente et al. 2010, Abou-Mansour et al. 2015).

(±) nigrosporione (7): \(^1\)H-NMR (400 MHz) and ESI/MS (+) data are in agreement with those previously reported (Wu et al. 2018).

3.2.7 Preparation of Extracellular Polysaccharides (EPSs)

The lyophilised culture filtrates of *N. australe* DAR79506 were dissolved in 300 ml of ultrapure Milli-Q water, cooled at 5°C and mixed with 5 volumes of absolute cold (-20°C) EtOH (1.5 L) and left overnight at -20°C. The resulting precipitate was collected by centrifugation (7000 rpm at 5°C for 45 min), dissolved in ultrapure Milli-Q water (300 ml) and precipitated again with absolute cold EtOH (1 L) as described above. After 24 h, the resulting precipitate was collected by centrifugation as described above, dissolved in a minimal amount of ultrapure Milli-Q water (30 ml), centrifuged again as described above and dialysed (cut-off 3500 Da) for two days against a large volume of water. The content was lyophilised to yield a crude EPSs fraction (47 mg). The same protocol was applied to the culture filtrates of *N. luteum* DAR81016 and *N. parvum* DAR80004, yielding 260 mg and 57 mg, respectively.

3.2.8 Phytotoxicity Bioassay

Neoantraquinone (3) and \(p\)-cresol (4) were assayed as previously reported (Reveglia et al. 2018b). However, the amount of nigrosporione (7) was very low and not sufficient for bioassay. Compounds 3 and 4 were dissolved in 40 μl of MeOH, and the volume was adjusted to 1 ml in sterile distilled water (SDW). The bioassays were conducted at two different concentrations, \(10^{-3}\) M and \(2.5 \times 10^{-3}\) M. Disease-free grapevine leaves were harvested from the fifth node of glasshouse grown *V. vinifera* cv. Shiraz, and the petiole of each leaf was immersed in a vial containing 1 ml of each of the solutions for 20 h, placed in a growth chamber with 12 h light/12 h darkness period at 28°C. SDW with 4% MeOH was used as a negative control. The leaves were then transferred to a new vial with 2 ml SDW, placed in a growth chamber with 12 h light/12 h darkness period at 28°C and maintained for an additional 28 h period. Presence of necrotic spots, wilting, distortion of the
lamina of the leaf surface were visually recorded after 48 h. Each experiment was conducted in triplicate.

The EPSs from *N. australis* DAR79506, *N. luteum* DAR81016 and *N. parvum* DAR80004 were tested at concentrations of 0.1, 0.5 and 1 mg ml<sup>-1</sup> on disease-free grapevine leaves harvested from the fifth node of glasshouse grown *V. vinifera* cv. Shiraz with six replicates for each dilution. Petioles of each leaf were immersed in 1 ml of solution containing the EPSs until complete absorption. Subsequently, the leaves were transferred into distilled water after 24 h. Symptoms on grapevine were visually recorded as above after 72 h. Negative control leaves were immersed in distilled water instead of EPSs.

3.3 Results

3.3.1 Phytotoxic Metabolites Produced by *N. australis* DAR79506

The purified organic extract of *N. australis* resulted in five amorphous solids. The preliminary <sup>1</sup>H NMR investigation permitted the identification of (R)-mellein (1, Figure 3.1) and tyrosol (2, Figure 3.1), that were previously isolated (Reveglia et al. 2018c) from this isolate. Compound 3 appeared to have a red colour and after an extensive 1D and 2D <sup>1</sup>H and <sup>13</sup>C NMR analyses (Table 3.1) it was characterised as a new anthraquinone and named neoanthraquinone (3, Figure 3.1). Neoanthraquinone has the molecular formula C<sub>18</sub>H<sub>16</sub>O<sub>4</sub> as deduced from its HRESI MS spectrum that was consistent with nine hydrogen deficiencies. Furthermore, the HRESI MS showed a significant protonated cluster [M + H]<sup>+</sup> and a sodium cluster at [M + Na]<sup>+</sup> at m/z 297.1127 and 319.0946, respectively. These data together with an extensive study of its <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra (Table 3.1) permitted the assignment of a symmetrical structure to compound 3. The <sup>1</sup>H NMR showed the presence of three singlets. The first at δ 6.25 (H-4 and H-5), typical chemical shift values, for aromatic protons located in ortho to a hydroxy group. The other singlets were assigned to the methyl groups bonded to an aromatic ring at δ 2.33 (Me- C1,8) and δ 1.95 (Me- C2,7) (Pretsch et al. 2000). The presence of the anthraquinone system was also consistent with the band typical for a hydroxyl group, conjugated carbonyl and aromatic group observed in the IR spectrum and the absorption maxima recorded in the UV spectrum (Nakanishi and Solomon 1977, Pretsch et al. 2000). The couplings observed in the HSQC spectrum (Berger and Braun, 2004) allowed the assignment of the chemical shifts to the protonated carbons of <sup>13</sup>C NMR spectrum (Table 3.1) and in particular the signal at δ 118.9 to C-4 and C-5, and that at δ 14.06 to CH<sub>3</sub>-C1,8, and finally that at δ 8.1 to CH<sub>3</sub>-C2,7 (Breitmaier and Voelter 1987). The couplings observed in the HMBC
spectrum (Table 3.1) between C-1,8 and H-4,5 and the methyl group CH₃-C1,8; C-2,7 and the methyl group CH₃-C2,7; C-10 and H-4,5; C-8a,8b with H-4,5 and CH₃-C2,7 allowed the assignment of the chemical shifts to the carbonyls, quaternary aromatic and olefinic sp2 carbons and namely the signals at δ 184.0, 178.8, 165.4, 158.9, 152.1, 129.7, 116.7 to C-10, C-9, C-3,6, C-1,8, C-8a,8b, C-4a,4b, and C-2,7, respectively. Thus, 3 was formulated as 3,6-dihydroxy-1,2,7,8-tetramethylanthracene-9,10-dione.

Table 3.1. ¹H and ¹³C NMR data of Neoanthraquinone (2)ᵃᵇ

<table>
<thead>
<tr>
<th>Position</th>
<th>δC ᶜ</th>
<th>δH</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8</td>
<td>158.9 C</td>
<td></td>
<td>H-4,5, CH₃-C1,8</td>
</tr>
<tr>
<td>2,7</td>
<td>116.7 C</td>
<td></td>
<td>CH₃-C2,7</td>
</tr>
<tr>
<td>3,6</td>
<td>165.4 C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4,5</td>
<td>118.9 CH</td>
<td>6.25(2H) s</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>178.8 C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>184.0 C</td>
<td></td>
<td>H-4,5</td>
</tr>
<tr>
<td>4a,4b</td>
<td>129.7 C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8a, 8b</td>
<td>152.1 C</td>
<td></td>
<td>H-4,5, CH₃-C2,7</td>
</tr>
<tr>
<td>CH₃-C1,8</td>
<td>14.06 CH₃</td>
<td>2.33 (6H) s</td>
<td></td>
</tr>
<tr>
<td>CH₃-C2,7</td>
<td>8.1 CH₃</td>
<td>1.95 (6H) s</td>
<td></td>
</tr>
</tbody>
</table>

ᵃThe chemical shifts are in δ values (ppm) from TMS.
ᵇ2D ¹H,¹H (COSY) and 2D ¹³C,¹H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.
ᶜMultiplicities were assigned by DEPT spectra.

Finally, the comparison of the spectroscopic data recorded for compound 5 with those already reported in literature allowed the assignment of the p-cresol structure (Kasthuraiah et al. 2004).
3.3.2 Phytotoxic Metabolites Produced by *N. luteum* DAR81016

(R)-mellein, tyrosol, (3R,4S)- and (3R,4R)-(-)-4-hydroxymellein (1,2,5,6 Figure 3.1) were isolated from the organic extract of *N. luteum* and their structure was confirmed by $^1$H NMR and ESIMS(+) (Kimura and Tamura 1973, Djoukeng et al. 2009, Evidente et al. 2010, Abou-Mansour et al. 2015). These data confirm those previously obtained in the preliminary analyses of the organic extracts of Australian *Botryosphaeriaceae* isolates (Reveglia et al. 2018c).

From the purification of other chromatographic fractions, neoanthraquinone (3, Figure 3.1) was identified. Moreover, compound 7 was extensively studied by 1D and 2D $^1$H and $^{13}$C NMR spectroscopy and by comparing the data with those in the literature it was recognised as nigrosporione (Wu et al. 2018). This result was further confirmed from its HRESI MS spectrum. When the optical specific rotation was measured, 7 did not show optical activity suggesting that it was isolated as a racemic mixture.

3.3.3 Phytotoxic Metabolites Produced by *N. parvum* DAR80004

The purification of the organic extracts obtained from culture filtrates of *N. parvum* results in four pure metabolites. The purified compounds were identified as (R)-mellein, tyrosol, (3R,4S)- and (3R,4R)-(-)-4-hydroxymellein (1,2,5,6 Figure 3.1) by comparing their spectroscopic properties ($^1$H NMR and ESIMS(+) with those previously reported in the literature (Djoukeng et al. 2009, Evidente et al. 2010, Abou-Mansour et al. 2015).
3.3.4 Bioassays of neoantraquinone (3) and p-cresol (4)

p-cresol (4) showed very low phytotoxicity when assayed at 2.5 x 10^{-3} M with only small and localised necrotic spots observed (Figure 3.2a) while no phytotoxic effects were visible at 10^{-3} M (Figure 3.2 b). Neoantraquinone (3) showed phytotoxic effects and this was dependent on the concentration. When assayed at 10^{-3}, compound 3 caused large necrotic spots localised close to the petiole (Figure 3.2 c). However, severe shriveling and distortion of the leaf lamina were observed when tested at 2.5 x 10^{-3} M (Figure 3.2 d). No phytotoxic symptoms were observed in the leaves that received the negative control treatment (Figure 3.2 e).

![Figure 3.2 Phytotoxic effects of: a) p-cresol at 10^{-3} M; b) p-cresol at 2.5 x 10^{-3} M; c) neoantraquinone at 10^{-3} M; d) neoantraquinone at 2.5 x 10^{-3} M; e) negative control (sterile distilled water).](image)

3.3.5 Phytotoxic Exopolysaccharides produced by Neofusicoccum spp.

The crude EPSs precipitated from the culture filtrates of *N. australe* DAR79506, *N. luteum* DAR81016 and *N. parvum* DAR80004 were initially analysed by $^1$H NMR. The typical signals of anomeric protons as well as other protons typical of carbohydrates were recognised in the spectra while these EPS were still in mixture (Pretsch et al. 2000), confirming their polysaccharide nature. When assayed on detached grapevine leaves, phytotoxic effects differed among species and concentrations. Overall results showed correlations between EPSs concentrations with phytotoxicity where the higher concentration of EPSs resulted in the greatest phytotoxicity.
No phytotoxic effects were observed for all the crude EPSs tested at 0.1 mg ml⁻¹. EPSs produced by *N. australe* showed small and localised necrotic spots at 0.5 mg ml⁻¹ (Figure 3.3a), while slight withering and dried leaf margins were observed at 1 mg ml⁻¹ (Figure 3.3b). Distortion of the leaf margin with necrotic spots was observed for EPS of *N. luteum* tested at 0.5 mg ml⁻¹ (Figure 3.3c). The same EPSs tested at 1 mg ml⁻¹ showed severe shriveling and distortion of the leaf (Figure 3.3d). The EPSs produced by *N. parvum* caused severe wilting of the leaf at both concentrations (Figure 3.3e and f). No phytotoxic symptoms were observed in the leaves that received the negative control treatment (Figure 3.3g and h).

![Figure 3.3 Phytotoxic effects of: EPSs from *Neofusicoccum australe* assayed at 0.5 mg ml⁻¹ (a) and 1 mg ml⁻¹ (b); EPSs from *N. luteum* assayed at 0.5 mg ml⁻¹ (c) and 1 mg ml⁻¹ (d); EPSs from *N. parvum* assayed at 0.5 mg ml⁻¹ (e) and 1 mg ml⁻¹ (f); negative control (sterile distilled water) (g and h).](image)

### 3.4 Discussion

This study represents the first comparison of the production of extracellular PMs and EPSs produced by *Neofusicoccum* spp. involved in BD. To date, no direct comparison of the extracellular phytotoxic compounds produced by different *Neofusicoccum* spp. is available in literature. The re-isolation of (R)-mellein and tyrosol from *N. australe* and melleins 1, 6 and 7 from *N. luteum* confirm the capability of these isolates to produce these compounds (Reveglia et al. 2018c). Furthermore, these PMs were isolated from the organic extract of *N. parvum* DAR80004. Melleins are common PMs produced by several pathogens involved in different diseases of agricultural crops including grapevine (Devys et al. 1980, Cabras et al. 2006, Djoukeng et al. 2009, Evidente et al.
In particular, \((R)\)-mellein has been isolated from different Botryosphaeriaceae species involved in trunk diseases and its biological activities were assayed on different hosts and under different conditions (Evidente et al. 2010, Ramírez-Suero et al. 2014, Masi et al. 2018a). Furthermore, the amount of \((R)\)-mellein detected in the culture media appears to be proportional to the aggressiveness of N. parvum and D. seriata (Ramírez-Suero et al. 2014). A more recent study has suggested that \((R)\)-mellein, together with \((+)-\)terremutin might assist the pathogens in overcoming the grapevines natural defense mechanism, weakening the plant immunity and favouring the disease (Trotel-Aziz et al. 2019). All these investigations suggest that \((R)\)-mellein could have an important role in the development of the disease caused by the pathogenic fungi. However, to support the in vitro results, reported in other studies, in planta experiments should be performed to gain definitive data on \((R)\)-mellein’s biological function.

The isolation of a novel phytotoxic anthraquinone, here named neoanthraquinone (3), further emphasise that isolates of N. australe have high metabolic variability (Andolfi et al. 2012). Anthraquinone represents a well-known class of natural compounds produced by plants and fungi. They are biosynthesised primarily through the acetate-mevalonate pathways (Dewick, 2002). Several anthraquinones have been isolated from different microorganisms including bacteria and fungi (Richardson et al. 1988, Gessler et al. 2013). Different anthraquinones have been isolated from organic extracts of Trichoderma spp. including pachybasin, chrysophanol, emodin and \(\omega\)-hydroxypachybasin (Liu et al. 2009). More recently, three new anthraquinone derivatives, named lentiquinones A, B, and C, and the known lentisone were isolated from Ascochyta lentis and assayed for their phytotoxic activity on host and non-host plants (Masi et al. 2018b).

The phytotoxic effect showed by neoanthraquinone (3) is not unexpected since many anthraquinones exhibit antibacterial, antiparasitic, insecticidal, fungicidal and antiviral activity (Gessler et al. 2013, Sugawara et al. 2019). The activity of these compounds is usually related to the presence of a quinoid structure, allowing the anthraquinones to participate in redox reactions. Furthermore, the aromatic rings create an electron-rich environment that traps the ROS, exhibiting scavenging properties against free radicals (Choi et al. 2000, Locatelli et al. 2011). Ueno et al. (1995) also suggested that the toxic effects of many anthraquinones may be due to the formation of semiquinone radicals. Considering their redox-properties, it may be possible that pathogenic fungi produce these PMs to protect themselves from the oxidative stress caused by the plant immune system.
\( p\)-cresol (4), is a very common naturally occurring phenol and has been recently isolated from the culture of \textit{Clostridium difficile}, and showed bacteriostatic activity against microorganisms including \textit{Escherichia coli}, \textit{Klebsiella oxytoca} and \textit{Bacteroides thetaiotaomicron} (Passmore et al. 2018). However, this is the first time that \( p\)-cresol was isolated as PMs from a pathogen involved in BD and was assayed on grapevine leaves showing only weak phytotoxic effect.

\((\pm)\) Nigrosporione (7) has been recently reported from \textit{Nigrospora sphaerica} isolated from \textit{Oxya chinesis} (Wu et al. 2018) also in epimeric mixture. The isolation of natural compounds in epimeric mixture has been already reported in a few cases from pathogenic fungi (Masi et al. 2014, Masi et al. 2017). However, Wu et al. (2018) purified the racemic mixture by chiral HPLC and assigned the absolute configuration by ECD and VCD. Furthermore, they tested the antifungal and antibacterial activity on different fungi and bacteria. They showed high antifungal activity and were only slightly active against bacteria (Wu et al. 2018). PMs with a cyclopentanone moiety have already been isolated from different microorganisms and they showed a different type of biological activity including phytotoxicity (Greulichi et al. 1995, Kozlovsky et al. 2003, Lübken et al. 2004). For those that showed phytotoxicity such as coronatine, the main hypothesis on their mode of action is that the cyclopenanone part of the compound might interfere with the jasmonate pathway of the plant, disrupting the plant defense system (Greulichi et al. 1995). Unfortunately, \((\pm)\) nigrosporione was isolated in very low amounts, thus, was insufficient to perform any phytotoxic bioassay on grapevine leaves. Future studies involving the re-isolation of this compound in higher amounts will further clarify its phytotoxic activity on hosts and non-host plants.

Pathogenic fungi involved in GTDs are capable of producing high molecular weight phytotoxins, including extracellular proteins and EPSs (Sparapano et al. 2000, Luini et al. 2010, Andolfi et al. 2011, Bénard-Gellon et al. 2015). \textit{P. chlamydospora} and \textit{P. minimun}, two fungal pathogens involved in the Esca complex were known to produce EPSs that showed phytotoxicity when assayed on grapevine leaves (Sparapano et al. 2000). These investigations led to the development of a rapid and specific method for the detection of EPSs in the leaves of Esca-affected grapevines using polyclonal antibodies. EPSs were unequivocally identified in diseased vines using flow cytometry (Andolfi et al. 2009). More recently, the capability of \textit{Botryosphaeriaceae} spp. to produce EPSs involved in GTDs have been investigated. Those produced by different \textit{Lasiodiplodia} spp. isolated in Brazil and those produced by \textit{N. parvum} have been studied for their phytotoxicity on grapevine leaves and tobacco leaves. EPSs from \textit{N. parvum} induced reddening and withering of the leaves. However, high variation in symptom expression between replicates was observed
Furthermore, the authors assigned the structure of a branched mannan to a purified EPS from *N. parvum*, essentially using NMR spectroscopy (Cimmino et al. 2016).

The results reported in this chapter represent the first report of EPSs produced by isolates of *N. australis* and *N. luteum* involved in BD. The results of the phytotoxic bioassay highlighted difference in toxicity among the species. EPSs from *N. parvum* appeared to be the most phytotoxic regardless the tested concentration. The phytotoxic effect of the EPSs is usually associated with their ability to compromise the nutrient uptake creating a physical barrier in the vessels of the plant (Sparapano et al. 2000). Differences in the phytotoxicity observed for these EPSs could reflect variation in their chemical structure. Indeed, high branched EPSs may reduce the nutrient uptake more rapidly resulting in a quicker wilting of the leaf. Future investigations should be focused on the purification and structural characterisation of the EPSs in order to shed light on the possible correlation between chemical structure and phytotoxic activity.

In conclusion, the results arising from this study contribute to knowledge on extracellular compounds that could play a potential role in the virulence of the three species assessed. Australian *Neofusicoccum* spp. produce PMs and EPSs that showed different degrees of phytotoxicity, confirming the previous results obtained in the preliminary analysis discussed in Chapter 2 (Reveglia et al. 2018c). The structural characterisation of these compounds may assist in the development of *in planta* experiments that could also assist in investigating a synergistic phytotoxic action between the different PMs, EPSs and extracellular proteins secreted by *Botryosphaeriaceae* spp. Furthermore, all three *Neofusicoccum* spp. produced various melleins as the main metabolites and they are typical PKs produced by the Botryosphaeriaceae (Masi et al. 2018a). However, production of specific PMs have been observed for the different species. These data could be a useful starting point for future investigation with the aim of carrying out metabolic profiling of different isolates of the different *Neofusicoccum* spp. involved in BD. Data arising from this investigation could be useful for: i) finding correlations between pathogen virulence and PMs produced; ii) finding a specific biological marker(s) for the disease; iii) using the metabolic profile in the identification of fungal species, and assisting the fingerprinting technique commonly used. However, to carry out such investigation, a high number of fungal isolates, possibly from different locations, vineyards and country are needed. Improving our knowledge on the PMs produced by *Neofusicoccum* spp. could provide additional insight into their biology and could potentially lead to the development of sustainable control methods for BD.
Chapter 4 – Production and translocation of *Botryosphaeriaceous* phytotoxins in naturally- and artificially infected vines

4.1 Introduction

Botryosphaeria dieback symptoms are normally categorised into internal symptoms including brown streaks and wedge-shaped discolorations in the wood, and external symptoms including death of the canes, shoots and buds, stunting of shoots, delayed bud burst and foliar symptoms (Larignon et al. 2001, Úrbez-Torres 2011, Pitt et al. 2013). The appearance of foliar symptoms in grapevines infected with GTDs including Esca complex (Mugnai et al. 1999, Surico et al. 2008) and ED (Carter 1991, Sosnowski et al. 2007, Rolshausen et al. 2008) is common in European vineyards. Foliar symptoms may be associated with the production of PMs by the fungi involved in the disease (Masi et al. 2018).

The first study that associated the expression of foliar symptoms with the production of PMs was conducted by Tey-Rulh et al. (1991) for ED. Different PMs were isolated from the culture filtrate of *E. lata* and eutypinol was detected in inflorescences and in the sap of diseased plants. The detection of this molecule beyond the inoculation point suggested that it could be translocated without being detoxified and was responsible for the development of foliar symptoms (Tey-Rulh et al. 1991). However, subsequent investigations conducted on wood extracts of diseased vines showing ED symptoms were unable to detect any of the phytotoxic metabolites produced by the causal organism, *E. lata* *in vitro* (Mahoney et al. 2005, Lardner et al. 2006, Rolshausen et al. 2008). Based on these results, the authors suggested that the fungal metabolites were not translocated but they may interact with the wood at the point of infection, disrupting the vascular structure. To date, the role of PMs in ED foliar symptoms is still unclear and requires further investigation.

Fungi involved in the Esca complex also produced PMs that belong to the class of naphthoquinones (Andolfi et al. 2011, Masi et al. 2018). The mode of action of these toxins may be related to their oxidant property that can play an important role in the interaction with the plant defence system. Furthermore, the tiger-stripe symptoms on leaves, commonly associated with Esca infected grapevines may be an indirect effect of the toxins produced by the fungi that colonise the wood.
causing physiological changes (Andolfi et al. 2011). It is possible that the foliar symptoms were a stress response of the plant and not a direct effect of the PMs released in the trunk (Andolfi et al. 2011, Masi et al. 2018). However, no definitive response to the role played by PMs for the Esca complex is available to date.

Investigations on the relationship of PMs and the expression of foliar symptoms are further complicated because the appearance of foliar symptoms appear to vary depending according to geographic location, season, climatic conditions, cultivar and age of the vines (Sosnowski et al. 2011, van Niekerk et al. 2011, Murolo and Romanazzi 2014). Furthermore, several investigations reported a relationship between water stress, rainfall and the expression of foliar symptoms in Esca and ED infected grapevines (Sosnowski et al. 2007, Larignon et al. 2009, Serra et al. 2018, Calzarano et al. 2019).

A number of studies have been reported on the phytotoxins produced by pathogens involved in BD (Martos et al. 2008, Djoukeng et al. 2009, Evidente et al. 2010, Abou-Mansour et al. 2015). The relationship between Botryosphaeriaceae infection and the expression of foliar symptoms appears to be complex where they were reported in some cultivars in the northern hemisphere (Mondello et al. 2018). However the foliar symptoms have never been observed in Australian vineyards to date (Billones-Baaijens et al. 2019). The PMs produced by Botryosphaeriaceae pathogens have also been detected in infected wood showing BD and Esca symptoms. Abou-Mansour et al. (2015) analysed the infected wood from four grapevines in order to determine whether the common phytotoxins identified in an in vitro culture of N. parvum were also produced in planta. Two toxic metabolites: (R)-mellein and (+)-terremutin were detected in the extracts of wood samples exhibiting brown stripes and black streaks. To date, this is the only study to report PMs produced by N. parvum in grapevines showing Esca and BD symptoms (Abou-Mansour et al. 2015).

In the previous chapters (Chapter 2 and 3), the ability of Australian Botryosphaeriaceae isolates to produce PMs were investigated in vitro. These results represent the first comprehensive investigation of PMs isolated and characterised from Australian isolates of the Botryosphaeriaceae. The isolations and characterisation of PMs secreted by fungi may assist in understanding the life cycle and infection processes of the pathogens. However, it is important to highlight that the chemical and biological characterisation of these compounds in vitro provides only partial information on their role in plant-pathogen interactions and development of the disease. Gaining
insight into this interaction may be crucial in understanding the role of PMs in the virulence of the pathogens and symptom expression of the disease.

The main objective of this Chapter was to investigate the production and translocation of PMs in naturally-infected and artificially-inoculated vines by Botryosphaeriaceae species using molecular and analytical chemistry techniques.

4.2 Materials and methods

4.2.1 Artificially inoculated vines

4.2.1.1 Grapevine varieties

Vitis vinifera cvs. Chardonnay and Cabernet Sauvignon, two of the most commonly grown varieties in Australian vineyards (Wine Australia 2018) were selected for the glasshouse experiment. Dormant, disease-free cuttings for each variety were collected in a commercial vineyard in Harden, New South Wales (NSW) in June 2017 and stored at 4°C for 4 weeks until rooting. All cuttings were rooted in plastic trays containing perlite and the trays were placed on heat pads at 30°C for 4 weeks to facilitate rooting. The rootlings were planted in 10 L pots containing commercial garden soil. All vines were maintained inside the glasshouse (17-27°C) and watered every 12 h for 5 minutes (8 L/h) with an automatic dripper system for 6-12 months until assessment.

4.2.1.2 Fungal isolates used for inoculation

The three Botryosphaeriaceae species: a) D. seriata H141a; b) S. viticola DAR78870 and; c) Do. vidmadera DAR78993 for which PMs were previously characterised (Reveglia et al. 2019, Reveglia et al. 2018b, Reveglia et al. 2018c) were used for inoculating the glasshouse vines. For inoculations, wounds were created in the middle internode of each vine using a flame-sterilised sterile cork borer (4 mm). Mycelial plugs (4 mm) cut from the margins of 4 day-old colonies of the selected species grown on potato dextrose agar supplemented with Chloramphenicol (500 mg L⁻¹) (PDA-C), were inserted into the wounds and sealed with Parafilm (Bemis, USA). Sterile non-colonised plugs of PDA-C were used as negative controls. The inoculated vines for each variety were arranged in a randomised complete block design (RCBD) at nine replications per inoculum x variety combination.
4.2.1.3 Assessment of inoculated rootlings

Representative samples of inoculated rootlings were assessed at 6 months (May 2018) and 12 months (November 2018) post inoculation (PI). At 6 months PI, three vines per treatment were randomly selected and the trunks were cut at the base and shoots were trimmed off. The bark surrounding the inoculation point was removed and lesions were measured using a digital caliper (Workzone, Australia). Tissue samples from each vine were collected as shown in Figure 4.1. Three different samples were collected from each vine: a) trunk sections with visible lesions including the inoculation point (IP); b) 2 cm trunk sections cut above and below the necrotic lesions (A,A); c) 2 cm trunk sections above and below section A and A’ (B,B’). The samples were surface-sterilised for 2 min in 70% ethanol and rinsed three times with sterile deionised water (SDW) before cutting longitudinally to obtain four quarters of each section. One quarter of the section was used for isolation of the pathogen while the remaining sections were stored at -80°C and used for DNA and toxin extractions.

Figure 4.1. A diagram of an inoculated vine showing the positions and sizes of tissue samples collected and used for analysis.
4.2.1.4 Fungal isolation from inoculated wood

For isolations, representative samples from surface-sterilised trunk sections collected from different parts of the inoculated vines (Figure 4.1) were plated onto PDA-C. Plates were incubated at 25°C in total darkness and observed for growth of *Botryosphaeriaceae* species for 4-7 days. The re-isolated pathogens were identified using morphological and molecular methods.

4.2.2 Naturally-infected vines

4.2.2.1 Sample collection

Wood samples (cords and trunks) exhibiting BD and cankers were collected from 20-24 year old grapevines from three vineyards in NSW, Australia in June-August 2017. Four to five wood pieces (10-20 cm) with necrotic lesions and non-necrotic tissues 20-30 cm away from the necrotic ones were cut from three different vines in Harden (cv. Shiraz), Borambola (cv. Shiraz) and Tumbarumba (cv. Chardonnay) and stored at 4°C for 2 weeks until processed.

4.2.2.2 Fungal isolation from naturally-infected vines

For isolations, the bark was removed from all wood to expose the wood with necrotic lesions and further cut into ~1 cm sections (20-24 pcs) with each section containing necrotic and healthy wood. All sections were surface-sterilised following the methods described for the inoculated vines. Approximately 10-12 pcs of the surface-sterilised tissues were stored at -80°C for DNA and toxin extractions. The remaining four sections were plated onto PDA-C for a total of three plates per vine. Plates were incubated at 25°C for 4-7 days and observed for growth of *Botryosphaeriaceae* species. All *Botryosphaeriaceae* isolates were identified by morphological and molecular methods.

4.2.3 DNA extractions from fungal mycelia

Crude DNA samples were extracted from the mycelia for all the isolated fungi using PrepMan Ultra and following the manufacturer’s instructions. Mycelium (~100 mg) was scraped from the edge of the colony for each isolate using a sterile pipette tip and transferred into a sterile 1.5 ml tube containing 100 µl of the PrepMan Ultra preparation reagent. Mycelial suspensions were vortexed for 30 s and incubated at 95°C in a heat block for 10 min. The tubes were centrifuged for 2 min at 3,220 × g and 50 µl of the supernatant was transferred to a new sterile 1.5 ml tube and stored at –20°C until used for PCR.
4.2.4 DNA extraction from wood

Wood samples stored in -80 °C were freeze-dried (Christ, John Morris Scientific, USA) for 24-36 h. Dried samples were homogenised at 20 Hz for 2 min 30 s using 10 ml grinding jars attached to a TissueLyser (Qiagen, USA). The ground wood (100 mg) was transferred into a sterile 2 ml tube for the DNA extraction while the remaining ground wood was stored in a separate tube for toxin extractions. DNA was extracted from wood samples using the methods described by Pouzolet et al. (2013) with some modifications. The CTAB (Cetyl Trimethyl Ammonium Bromide) extraction buffer from Doyle and Doyle (1987) was prepared using 100 mM Tris-HCl ((tris(hydroxymethyl)aminomethane hydrochloride, Sigma-Aldrich, USA), 20 mM EDTA (Ethylenediaminetetraacetic acid), 1.4 mM NaCl, 2% CTAB, PVP (polyvinyl pyrrolidone), 0.5% β-mercaptoethanol (Sigma-Aldrich, USA) and 0.4% of RNAse A (Doyle and Doyle 1987). The CTAB buffer (1 ml) was added to each tube containing 100 mg of ground wood and gently mixed by pipetting. The mixture was incubated at 65°C for 1 h using a heat block. After incubation, 500 µl of chloroform/isoamyl alcohol (24:1, Sigma Aldrich, USA) was added and the tube was inverted 10 times, incubated on ice for 5 min and then centrifuged at 4°C for 10 min at 2300 × g. Approximately 420 µl of the lysate was pipetted into a QIAshredder spin column placed in a 2 ml collection tube from the Qiagen DNeasy Plant DNA extraction kit and centrifuged for 2 min at 20,000 × g. The subsequent steps were performed using the buffers, materials and protocol from the DNeasy Plant DNA extraction kit. All DNA samples were eluted to a final volume of 100 µl using the Qiagen AE buffer. All DNA samples were quantified using a Quantus™ Fluorometer (Promega, USA) prior to qPCR.

DNA was further extracted from the necrotic tissues collected from potted vines (cv. Chardonnay) inoculated six months prior with *N. parvum* DAR78998 following the methods described above. This isolate was found to be highly virulent in a separate experiment (Pitt et al. 2013) and is known to produce (R)-mellein *in vitro*. The DNA extracted from these vines was included as positive controls for qPCR and toxin analyses.

4.2.5 Identification of isolated *Botryosphaeriaceae* by PCR

All *Botryosphaeriaceae* isolates recovered from the artificially-inoculated and naturally-infected vines were identified using PCR and DNA sequencing. To amplify the internal transcribed spacer (ITS) region of the ribosomal DNA of the pathogens, the PCR was performed using the universal
primers ITS1 and IT4 (White et al. 1990). Each 25 µl PCR reaction contained 1x PCR buffer, 0.4 µl for each of primer, 1.25 U of My TaqRed DNA polymerase and approximately 1-5 ng of DNA template. PCRs were performed using a thermal cycler (C100 Thermal cycler, Biorad Laboratories, Pty, Ltd., U.S.A.) under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of 30 s at 94°C, 45 s at 55°C, and 90 s at 72°C, with a final extension of 5 min at 72°C. Following amplification, 5 µl of the PCR products were visualised by gel electrophoresis using 1% agarose. Gels were stained using 1 × GelRed™ nucleic acid gel stain and visualised under UV light using a Gel Doc XR+ Imaging System (BIO-RAD Laboratories, Pty, Ltd.). PCR products were cleaned with FavorPrep Gel/PCR purification kit and sent for DNA sequence analyses at the Australian Genome Research Facility (AGRF; Sydney, NSW, Australia). All DNA sequences and chromatographs were analysed using the DNAMAN 5.2 (Lynnon Biosoft©) and Chromas Lite 2.1© (Technelysium PTY Ltd) software. All trimmed DNA sequences were analysed using the Basic Local Alignment Search Tool (BLAST) in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

### 4.2.6 Quantification of *Botryosphaeriaceae* spp. from wood samples by qPCR

The qPCR assay using *Botryosphaeriaceae* multi-species primers and hydrolysis probe (Table 4.1) developed by Billones-Baaijens et al. (2018) were used to detect and quantify *Botryosphaeriaceae* spp. from artificially-inoculated and naturally-infected vines.

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bot-BtF1</td>
<td>GTATGGCAATCTTCTGAACG</td>
<td>β-tubulin</td>
</tr>
<tr>
<td>Bot-BtR1</td>
<td>CAGTTGTTACCGGCRCCAGA</td>
<td>β-tubulin</td>
</tr>
<tr>
<td>Taq-Bot probe</td>
<td>5’/36-FAM/TCGAGCCCG/ZEN/GCACCATGGAT/3IBkFQ/3’</td>
<td>β-tubulin</td>
</tr>
</tbody>
</table>

*Integrated DNA technologies, Singapore.*

* Billones-Baaijens et al. 2018.

All qPCR assays were performed using a RotorGene 6000 (Corbett Life Science, Qiagen, USA) according to the methods developed by Billones-Baaijens et al. (2018). Each 20 µl reaction contained 10 µl of 2× GoTaq® Master Mix (Promega, USA); 500 nm each of the primers Bot-BtF1
and Bot-BtR1, 250 nm of Taq-Bot probe and 5 µl of template DNA at four technical replicates per sample. All qPCR analyses were performed using the following thermal cycling conditions: 2 min at 98°C, followed by 40 cycles of 98°C for 10 s, 60°C for 30 s and fluorescence detection at 60°C for 30 s. For each assay, two controls were included: 1) non-template control (H2O); b) standard (500 pg) Bot-Btub gBlock (Billones-Baaijens et al. 2018) at four technical replicates each.

For the artificially-inoculated vines, all necrotic tissue samples collected from the IP were first analysed followed by the tissues collected above and below the necrotic lesions (AA’). For AA’ tissues tested positive to Botryosphaeriaceae DNA, its subsequent BB samples were further analysed for a total of 32 and 36 samples for 6- and 12-months PI respectively. For naturally-infected vines, wood samples containing necrotic tissues were first analysed by qPCR. When the necrotic tissue sample was positive to qPCR, the healthy wood samples away from the same necrotic tissue was further analysed for a total of 18 samples overall.

To determine the amount of pathogen DNA that was amplified by each qPCR assay, previously developed standard curves (Billones-Baaijens et al. 2018b) were imported in the Rotor-Gene Q software (Version 2.3.1). The standard (Bot-Btub gBlock, 500 pg) that was included in each qPCR assay was used to calibrate the imported standard curve and the resulting regression equations were used to quantify the number of Botryosphaeriaceae β-tubulin gene copies in each reaction as previously described by Billones-Baaijens et al. (2018).

To calculate the number of copies of the Botryosphaeriaceae β-tubulin gene in each wood sample, the following formula was used:

\[ N = \frac{g(d \times c)}{T \times c} \]

Where:

N = calculated number of β-tubulin gene copies in one wood sample,

g = the mean number of gene copies detected by qPCR;

d = total gDNA extracted from 100 mg of wood (100 µl),

c = DNA concentration (µl)

T = the amount of DNA template (5 µl) in one reaction.
4.2.7 Statistical analysis

Data arising from the glasshouse experiment were analysed using IBM SPSS 24 software. All data were tested for homogeneity using Levene’s test at \( P \leq 0.05 \). For inoculated vines, univariate analysis of variance (ANOVA) was used to assess differences in lesion lengths and pathogen copies between varieties, inoculated pathogens and their interactions. \( (P \leq 0.05) \). All means were separated by pairwise comparison using least significant differences (LSD) at 5% significance level. Statistical analysis was not applied to the naturally-infected vines because of differences in the storage and sampling of the collected wood. The ANOVA tables are reported in Appendix C.

4.2.8 Detection of PMs from plant samples using LC-MS/MS

4.2.8.1 Chemicals and Standards

CHCl₃, MeOH and \( n \)-hexane were used for extraction of PMs from wood. Water 0.1% formic acid and ACN LC-MS grade were used for LC-MS/MS analysis. The (R)-mellein used as standard was isolated and purified from \textit{in vitro} culture of \textit{D. seriata} as previously reported (Reveglia et al. 2019). The spencertoxin used as a standard was isolated and purified from an \textit{in vitro} culture of \textit{S. viticola} as previously reported (Reveglia et al. 2018b). The protocatechuic alcohol used as a standard was isolated and purified from an \textit{in vitro} culture of \textit{Do. vidmadera} as previously reported (Reveglia et al. 2018c).

4.2.8.2 Testing of protocols for extraction of PMs from wood

Two published extraction protocols were tested for their suitability to extract toxins from wood samples. All the freeze-dried wood samples collected from the naturally-infected vines were extracted using both protocols. For the first protocol (Abou-Mansour et al. 2015), the toxin was extracted from 100 mg freeze-dried and finely ground wood using 1 ml of MeOH at 40°C with agitation for 1 h using a stirring hot plate. The extracts were pooled, and the solvent was reduced to 5 ml under a steam of nitrogen and then further extracted twice with 5 ml of \( n \)-hexane and dried under nitrogen flow. For the second protocol (Saviano et al. 2016) the freeze-dried ground tissue (1 g), was transferred into a 25 ml centrifuge tube, and 4 ml of MeOH, 1.7 ml of water and 4 ml of chloroform per gram of freeze-dried tissue (all solvents were at 4°C) were added to the tube, and vortexed for 30 s. The sample was gently stirred and mixed on ice for 10 min (the solution must be mono-phasic). Four millilitre of chloroform and 4 ml of water per gram of freeze-dried sample were added and the final mixture was vortexed and centrifuged at 3000 rpm for 15 min at 4°C. The methanol/water and chloroform fractions were separately collected in 2 ml glass vials, dried in a
vacuum at room temperature and stored at -80 °C until LC-MS/MS analysis. All organic extracts obtained from both protocols from naturally-infected vines (18 total) were analysed by LC-MS/MS and the results showed that the second protocol (Saviano et al. 2016) resulted in higher yields of (R)-mellein. This protocol was subsequently used to extract PMs from the wood samples (500 mg) collected from inoculated vines (72 in total).

4.2.8.3 LC-MS/MS analysis
Analyses were carried out using a 1290 Infinity II LC system (Agilent, U.S.A) hyphenated to an Agilent 6470 triple quadrupole (QqQ). The UPLC system included a binary pump and a cooled autosampler maintained at 15°C. Mass Hunter software was used to control the instruments and to acquire the data which were then processed for analysis.

The chromatographic separation was performed using a reverse phase column Phenomenex Luna 5.4 µm 250 × 4 mm i.d., protected by a security guard column Phenomenex maintained at 30°C. The mobile phase consisted of water 1% (v/v) formic acid (solvent A) and ACN (solvent B). The flow rate was 0.7 ml/min, the gradient system was initiated with 10% of solvent B for 2 min and reached 30% at 15 min, 80% at 25 min, isocratic until 27 min and 95% at 40 min. Samples were injected into the column with an injection volume of 20 µl.

The Agilent 6470 triple quadrupole (QqQ) was used in MRM mode with electrospray ionization (ESI) in positive ionization mode. The source and desolvation temperatures were respectively set at 350°C, Nebulizer, 40 psi; N₂ flow, 12 L min⁻¹. Capillary voltage was set at 3.5 kV in positive mode. The MRM transitions (precursor ion → daughter ions), fragmentor energy and collision energy for (R)-mellein, spencertoxin and protocatechuic acid were optimized using the Agilent Optimizer Software, and the optimized parameters shown in Table 4.2. For inoculated wood samples, the purified (R)-mellein; spencertoxin and protocatechuic acid obtained by Reveglia et al. (2019, 2018 b and c) were used as standards while only (R)-mellein was used as a standard for naturally-infected vines.
Table 4.2. Purified toxins used as standards for the LC/MS-MS and their corresponding optimised parameters.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Precursor Ion</th>
<th>Fragment Ions</th>
<th>Fragmentor</th>
<th>CV</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>((R))-mellein(^a)</td>
<td>179 ([M+H])^+</td>
<td>161, 133, 105</td>
<td>90</td>
<td>12, 16, 24</td>
<td>27.441</td>
</tr>
<tr>
<td>Protocatechuic</td>
<td>123 ([M\cdot H2O+H])^+</td>
<td>67.1, 55.1, 51.1</td>
<td>90</td>
<td>16, 24, 40</td>
<td>6.939</td>
</tr>
<tr>
<td>alcohol(^c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spencertoxin(^b)</td>
<td>283 ([M+K])^+</td>
<td>177.8, 118</td>
<td>90</td>
<td>44, 50</td>
<td>18.112</td>
</tr>
</tbody>
</table>

\(^a\) Purified from *in vitro* culture of *Diplodia seriata* (Reveglia et al. 2019).
\(^b\) Purified from *in vitro* culture of *Dothiorella vidmadera* (Reveglia et al. 2018c).
\(^c\) Purified from *in vitro* culture of *Spencermartinsia viticola* (Reveglia et al. 2018b).
4.3 Results

4.3.1 Artificially-inoculated vines

4.3.1.1. Wood symptoms
All inoculated vines collected at 6 months PI did not exhibit foliar symptoms or external necrosis and all plants showed normal development. However, when the bark was peeled from the vines and the trunks were cut longitudinally, vascular staining and discolouration of the wood close to the IP were observed for the samples inoculated with the pathogens. The lesions lengths were not significantly different between varieties or any of the inoculation treatments including the negative controls ($P > 0.05$; Figure 4.3 and 4.4). All three *Botryosphaeriaceae* species were re-isolated from their corresponding trunk sections with necrotic lesions, while no *Botryosphaeriaceae* pathogens were re-isolated from any of the negative control vines.

At 12 months PI, all vines also showed normal development and did not exhibit any external symptoms. However, vascular staining and discolouration of wood that progressed upward and downward from the inoculation point were also observed from the longitudinal sections (Figure 4.2). The lesion lengths for all treatments were significantly longer than those observed at 6 months PI (Figure 4.3). The overall lesion lengths differed significantly between varieties with Chardonnay being the most susceptible with mean lesion lengths of 20.2 mm which was significantly longer ($P \leq 0.05$) than Cabernet Sauvignon (17.3 mm). The overall lesion lengths also varied between inoculation treatments with lesions produced by *Do. vidmadera* (20.8 mm), *S. viticola* (20.4 mm) and *D. seriata* (19.4 mm) being significantly longer ($P \leq 0.05$) compared to the negative control vines (13.1 mm). There were no significant interactions between varieties and inoculation treatments based on lesion lengths ($P > 0.05$) (Figure 4.4).
Figure 4.2. Longitudinal sections of *Vitis vinifera* (cv. Chardonnay) vines 12 months post inoculation. a) negative control; and vines inoculated with (b) *Diplodia seriata* H141a (c) *Dothiorella vidmadera* DAR78993 and (d) *Spencermartinsia viticola* DAR78870

Figure 4.3. Overall mean lesion lengths caused by *Diplodia seriata* H141a, *Dothiorella vidmadera* DAR78993 and *Spencermartinsia viticola* DAR78870 on *Vitis vinifera* (cvs. Chardonnay and Cabernet Sauvignon) at 6- and 12-months post inoculation (PI). Bars with different letters for each inoculation period are significantly different at $P \leq 0.05$ LSD. Error bars represent standard error of the means.
Figure 4.4. Overall mean lesion lengths caused by Diplodia seriata H141a, Dothiorella vidmadera DAR78993 and Spencermartinsia viticola DAR78870 on Chardonnay and Cabernet Sauvignon at 6 and 12 months post inoculation (PI). Bars with different letters for each inoculation period are significantly different at \( P \leq 0.05 \) LSD. Error bars are standard error of the means.

4.3.2 qPCR analysis of inoculated wood samples

4.3.2.1 Symptomatic tissues at inoculation point

At 6 months PI, Botryosphaeriaceae DNA was detected by qPCR at the inoculation point (IP) of wood samples with necrotic tissues (IP) collected from vines inoculated with all three Botryosphaeriaceae species. The highest amount of pathogen DNA was detected from Chardonnay vines inoculated with S. viticola with significantly higher \( (P<0.05) \) Botryosphaeriaceae DNA than Chardonnay inoculated with D. seriata. The amount of Botryosphaeriaceae DNA from Chardonnay inoculated with Do. vidmadera was not significantly different from those inoculated with D. seriata or S. viticola (Figure 4.5). Botryosphaeriaceae DNA detected from all Cabernet Sauvignon infected vines was significantly lower compared to those from Chardonnay, regardless of species. Significant interaction between variety and species was observed \( (P < 0.05) \) which was associated with a significantly higher amount of Botryosphaeriaceae DNA from all Chardonnay vines compared to Cabernet Sauvignon. Botryosphaeriaceae DNA was not detected from any of the non-inoculated vines while the positive control sample \( (N. parvum) \) all tested positive to the Botryosphaeriaceae DNA (data not shown).
Figure 4.5. Overall mean of Botryosphaeriaceae β-tubulin gene copies detected from Vitis vinifera (cvs. Chardonnay and Cabernet Sauvignon) inoculated with Diplodia seriata H141a, Spencermartinsia viticola DAR78870 and Dothiorella vidmadera DAR78993 at 6 months post inoculation using quantitative PCR. Bars with different letters for each inoculation period are significantly different at $P \leq 0.05$ LSD. Error bars are standard error of the means. All non-inoculated control vines were tested negative to Botryosphaeriaceae DNA and were excluded in the graph.

At 12 months PI, Botryosphaeriaceae DNA were further detected by qPCR from necrotic wood samples (IP) excised from vines inoculated with either of the three species (Figure 4.6). The amount of pathogen DNA across treatments increased by 10-fold compared to the amount detected at 6 months PI. The highest amount of pathogen DNA was detected from Chardonnay vines inoculated with D. seriata with significantly higher amounts ($P<0.05$) compared to Chardonnay and Cabernet Sauvignon vines inoculated with S. viticola and Do. vidmadera. The lowest amount of pathogen DNA was detected from Cabernet Sauvignon vines inoculated with D. seriata and this was significantly lower than for all other inoculated vines. Significant interaction between variety and species was observed ($P < 0.05$) which was associated with the greatest amount of Botryosphaeriaceae DNA from Chardonnay and the lowest from Cabernet Sauvignon vines inoculated with D. seriata.
Figure 4.6. Overall mean of *Botryosphaeriaceae* β-tubulin gene copies detected from necrotic tissues at the inoculation point of *Vitis vinifera* (cvs. Chardonnay and Cabernet Sauvignon) vines inoculated with *Diplodia seriata* H141a, *Spencermartinsia viticola* DAR78870 and *Dothiorella vidmadera* DAR78993 at 12 months post inoculation using quantitative PCR. Bars with different letters for each inoculation period are significantly different at $P \leq 0.05$ LSD. Error bars are standard error of the means. All non-inoculated vines were negative to *Botryosphaeriaceae* DNA and were excluded in the graph.

### 4.3.2.2 Asymptomatic tissues (AA’ and BB’)

At 6 months PI, *Botryosphaeriaceae* DNA was further detected by qPCR from asymptomatic tissues (AA’) for some inoculated vines. For Chardonnay, *Botryosphaeriaceae* DNA was detected from asymptomatic tissues (AA’) in one out of three of the biological replicates of vines inoculated with *S. viticola* (69 copies) while *Botryosphaeriaceae* DNA was not detected from asymptomatic tissues of the *D. seriata* and *Do. vidmadera* inoculated vines. For Cabernet Sauvignon, one out of three of the biological replicates of vines inoculated with *D. seriata* (40 copies), *S. viticola* (440 copies) and *Do. vidmadera* (190 copies) showed a different number of copies of *Botryosphaeriaceae* DNA. Furthermore, all asymptomatic tissues (AA’) regardless of treatments were negative to *Botryosphaeriaceae* DNA by qPCR at 12 months PI. For those AA’ samples that were positive to qPCR, none of their subsequent samples BB’ sections were positive to pathogen DNA (data not presented).
4.3.3 Naturally-infected vines

All wood samples from three different vineyards in NSW showed dieback symptoms, cankers and typical wedge-shaped necrosis (Figure 4.7). Personal communications with vineyard managers indicate none of the vines showed foliar symptoms prior to sampling, regardless of the vineyard and variety.

Isolations from tissue samples collected from the vines for all three vineyards showed they were positive to *Botryosphaeriaceae* species (Table 4.3). *D. seriata* was the most prevalent species being present in three vineyards and six out of the nine vines sampled. In Harden, one vine was positive to *N. parvum*, while the other two vines were positive to *D. seriata* and *D. mutila*. In Tumbarumba, two vines were positive to *D. seriata* while the other vines were positive to both *D. seriata* and *N. parvum*. For Borambola, two vines were positive to *D. seriata* while one was positive to *B. dothidea*.
Table 4.3. *Botryosphaeriaceae* species isolated from naturally-infected vines from three vineyards in New South Wales, Australia.

<table>
<thead>
<tr>
<th>Location</th>
<th>Variety</th>
<th><em>Botryosphaeriaceae species</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Harden</td>
<td>Chardonnay</td>
<td><em>Neofusicoccum parvum</em></td>
</tr>
<tr>
<td>Harden</td>
<td>Chardonnay</td>
<td><em>Diplodia seriata</em></td>
</tr>
<tr>
<td>Harden</td>
<td>Chardonnay</td>
<td><em>D. mutila</em></td>
</tr>
<tr>
<td>Tumbarumba</td>
<td>Chardonnay</td>
<td><em>D. seriata</em></td>
</tr>
<tr>
<td>Tumbarumba</td>
<td>Chardonnay</td>
<td><em>D. seriata, N. parvum</em></td>
</tr>
<tr>
<td>Tumbarumba</td>
<td>Chardonnay</td>
<td><em>D. seriata</em></td>
</tr>
<tr>
<td>Borambola</td>
<td>Shiraz</td>
<td><em>D. seriata</em></td>
</tr>
<tr>
<td>Borambola</td>
<td>Shiraz</td>
<td><em>Botryosphaeria dothidea</em></td>
</tr>
<tr>
<td>Borambola</td>
<td>Shiraz</td>
<td><em>D. seriata</em></td>
</tr>
</tbody>
</table>

4.3.4 qPCR analysis of naturally-infected wood samples

All necrotic tissue samples from all vines obtained from three vineyards, tested positive to *Botryosphaeriaceae* DNA by qPCR (Figure 4.8). The greatest amount of pathogen DNA was detected from the three vines from Borambola, while the lowest amount of DNA was detected from all vines obtained from Harden and Tumbarumba. No *Botryosphaeriaceae* DNA was detected in any of the asymptomatic wood samples that were collected from the same vines. Statistical analysis was not applied to the naturally-infected vines because of differences in the storage and sampling of the collected wood.
Chapter 4

4.3.5 Selection of protocol for extraction of PMs from wood

A total of 36 organic extracts were obtained for testing two different protocols for the extraction of PMs: A) n-hexane/MeOH (Abou-Mansour et al. 2015); and B) water/MeOH/chloroform (Saviano et al. 2016). Protocol B was less time consuming and resulted in a higher amount of organic extracts. Analysis of the organic extracts using LC/MS-MS to detect (R)-mellein also showed that protocol B yielded a higher amount of the target metabolite. The peak of (R)-mellein detected in the extract obtained with Protocol B (Figure 4.9a) was 12-fold higher than the organic extract obtained with the protocol a (Figure 4.9b). Based on these results, Protocol B was further used for extracting PMs from inoculated vines.

Figure 4.8. Number of copies of pathogen DNA quantified by qPCR in naturally-infected vines from three vineyards in New South Wales, Australia. Error bars are standard deviation of the means.
4.3.6 LC-MS/MS analysis of naturally-infected wood samples

Unambiguous identification of \((R)\)-mellein from extracts of asymptomatic and symptomatic wood of the nine grapevine plants was accomplished according to its retention time, precursors ion 179 m/z [M+H]⁺ and fragment ions. The analysis of the extracts of symptomatic wood samples gave different results depending on the collected vines and vineyards. Only one out of three Chardonnay vines from Harden tested positive to \((R)\)-mellein, two out of three Chardonnay vines from Tumbarumba vineyard were positive while all the three Shiraz grapevines from Borambola vineyard were positive to \((R)\)-mellein. \((R)\)-mellein was not detected in any of the asymptomatic wood samples. The area of \((R)\)-mellein peak in the LC/MS-MS analysis was greatest for the vines with a high number of DNA copies of the pathogen in the trunk (Figure 4.10).
4.3.7 LC/MS-MS analysis of inoculated wood samples

At six months PI, none of the necrotic wood samples (IP) from infected vines were positive to the three target PMs. However, (R)-mellein was detected from the extracts of necrotic wood samples (IP) of both Chardonnay and Cabernet Sauvignon vines infected with *D. seriata* at 12 months PI. Furthermore, neither spencertoxin nor protocatechuic acid were detected from necrotic wood samples (IP) infected with *S. viticola* and *Do. vidmadera*, respectively, 12 months PI.

(R)-mellein was further detected from extracts obtained from the Chardonnay vine infected with *N. parvum* 78998 from a separate experiment and included in the test as a positive control. The peak of (R)-mellein detected from the *N. parvum* (DAR78998) infected vine was 2.4- fold higher than the amount detected in vines infected with *D. seriata* 12 months PI (Figure 4.11). All negative control vines and the asymptomatic tissues (AA’ and BB’) tested negative to all the PMs selected for the analysis.
4.4 Discussion

This is the first comprehensive study to investigate the production of PMs by *Botryosphaeriaceae* species in naturally-infected and artificially-inoculated vines using a multi-faceted approach. A previous study that investigated the production of PMs in planta only used at a limited number of naturally-infected vines (Abou-Mansour et al. 2015). This current study also represents the first study to use a combination of conventional plant pathology and molecular techniques to detect and quantify *Botryosphaeriaceae* DNA from artificially-inoculated and naturally-infected vines. This study showed that lesion length by itself was insufficient to show differences in disease severity and pathogen virulence at the early stage of infections (in this case, 6 months PI). Furthermore, negative control vines also exhibited necrosis near the IP similar to those vines inoculated with pathogens. The qPCR analysis, on the other hand, was able to quantify the amount of pathogen DNA between treatments and assessment periods while all negative control vines tested negative to the pathogen. It is important to highlight that qPCR primers and probes used in this study (Billones-Baaijens et al. 2018) are not species-specific, therefore, they cannot distinguish the pathogen at the species level. However, the isolation of pathogens from naturally-infected wood allowed the identification of *Botryosphaeriaceae* spp. and re-isolations of the pathogens from artificially-inoculated vines which resulted in the recovery of *D. seriata*, *S. viticola* and *D. vidmadera* from previously inoculated.
vines. Furthermore, all non-inoculated vines tested negative to the pathogens by re-isolations and qPCR.

The qPCR analysis of inoculated vines showed a significant difference in susceptibility between varieties to different *Botryosphaeriaceae* species. The number of DNA copies obtained from Chardonnay was higher than for Cabernet Sauvignon at 6 months PI, regardless of the inoculated pathogen. However, at 12 months PI, differences in susceptibility between the two varieties were only observed in vines inoculated with *D. seriata* and *S. Viticola*, while the two varieties showed similar susceptibility to *Do. vidmadera*. Furthermore, a higher amount of DNA copies of *D. seriata* were detected in Chardonnay compared to the other two species suggesting that some varieties may have different levels of susceptibility to different *Botryosphaeriaceae* species. The reduced susceptibility of Cabernet Sauvignon to some *Botryosphaeriaceae* species may be associated with the amount of stilbene polyphenols that are usually higher in red vine varieties. These compounds have fungistatic activity but their antimicrobial activity depends on the pathogen infecting the plant (Del Rio et al. 2004, Lambert et al. 2012). Thus, a higher concentration of stilbene polyphenols may assist the plant in limiting fungal infection (Lambert et al. 2012).

The qPCR analysis used in this study was also effective in quantifying pathogen DNA in symptomatic wood samples collected from naturally-infected vines. Wood samples from the Borambola vineyard contained the highest number of DNA copies that was 5-fold higher and 1.4-fold higher than the DNA from vines in Harden and Tumbarumba vineyards, respectively. However, due to differences in the storage and sampling of the collected wood, statistical analysis was not applied to this qPCR data. Overall, these results confirm that molecular techniques could be applied to determine and quantify the pathogens in field material. Future investigations will be oriented to developing a standard protocol for the collection of samples and storage in order to perform statistical analysis of the qPCR data and to highlight possible relationships between the number of DNA copies and severity of infection.

The LC/MS-MS analysis of the naturally-infected and artificially-inoculated vines showed that (R)-mellein can be detected in infected wood showing symptoms of BD, confirming the data previously reported by Abou-Mansour et al. (2015). The detection of PMs in infected plant tissue is an important result because this may provide insight into the involvement of PMs in the pathogenicity of fungal isolates and symptom development of the disease. The detection of target PMs in different plant tissues have been reported in other pathosystems. For example, Botrydial, a PM produced by
Botrytis cinerea, was detected in ripe fruits of Capsicum annum inoculated with the pathogen (Deighton et al. 2001). The study also found a correlation between the aggressiveness of the fungal isolates and the amount of botrydial detected in planta by LC-MS (Deighton et al. 2001). Helvolic acid and cerulenin were also detected in the organic extracts of rice grains infected with sheath rot caused by Sorocladium oryzae (Ghosh et al. 2002). The authors suggested that these metabolites may increase the pathogenicity and survival of S. oryzae by competing with other seed-borne fungi. Coronatine is a well-known fungal phytotoxin and was detected in various host plants infected by Pseudomonas syringae by an indirect competitive ELISA using the monoclonal antibody 11B8 (Zhao et al. 2001). The application of immunofluorescence microscopy and immunogold labelling showed that coronatine was present inside tomato cells, it was mobile in infected plant tissue and was detected in healthy tissue adjacent to the bacterial lesions.

(R)-mellein is a common phytotoxin and its contribution to the virulence of Botryosphaericeae spp. is strongly suspected (Trotil-Aziz et al. 2019). However, the involvement of (R)-mellein in symptom expression and in plant-pathogen interaction requires further investigation. In a recent study using in vitro plantlets, Trotil-Aziz et al. (2019) showed that (R)-mellein strongly suppresses the expression of genes involved in plant defence. Furthermore, their results also suggested that (R)-mellein may be accumulated in planta in its native chemical form. Furthermore, the application of a multi-faceted approach suggests a probable correlation between the amount of pathogen DNA in the wood and the area of the peak of the (R)-mellein in the chromatograms and is directly dependant on the amount in the wood. This correlation was more evident in Borambola vines which gave more intense peaks corresponding to (R)-mellein and the highest amount of pathogen DNA in the wood. Moreover, the amount of (R)-mellein detected in the wood infected with N. parvum six months PI was 2.4-fold higher than the amount detected in vines infected with D. seriata twelve months PI. These data further confirm those previously reported that the amount of (R)-mellein produced by N. parvum and D. seriata under in vitro conditions is proportional to the aggressiveness of the pathogens (Ramírez-Suero et al. 2014).

The detection of (R)-mellein only in the symptomatic tissues showing a high amount of pathogen DNA suggests that at least under these experimental conditions, the translocation of (R)-mellein to the foliage did not occur, but perhaps it performs its biological function in the trunk. Moreover, considering that the foliar symptoms have never been observed in Australian vineyards, the detection of (R)-mellein in the infected wood highlights that the production of PMs in planta by the pathogens may not be large enough amounts to result in BD foliar symptoms. Instead, they may be
caused by a combination of biotic and abiotic stress factors which require more in-depth studies. It is important to note that low levels of pathogen DNA were detected from non-necrotic tissues adjacent to the lesions from a few inoculated vines at 6 month PI, particularly for *S. viticola*. This result suggests that the pathogens were able to move endophytically beyond the lesions as latent pathogens similar to those reported by Billones-Baaijens et al. (2013). The absence of pathogen DNA on non-necrotic tissues adjacent to the lesions at 12 months PI suggests that as the infection advanced, these pathogens shifted from being latent to necrotrophic.

Fungi involved in GTDs can act as endophytes for several years before becoming pathogens, and many researchers have hypothesised that the abiotic conditions, in particular thermal and water stress, can trigger GTD symptom development (Serra et al. 2019). A comprehensive review on the influence of thermal and water stress on the incidence and severity of GTDs have been recently published by Songy et al. (2019). Based on the available data, the authors draw the following conclusions: i) cool and rainy summers favour the expression of foliar symptoms of Esca; ii) low water regimes in the soil appear to increase the woody symptoms, iii) the development of foliar symptoms is not strictly correlated with internal symptoms (Songy et al. 2019).

With a special emphasis on the foliar symptoms of Esca complex, field studies indicate that their expression and fluctuation appears to depend on plant phenology and water status. Indeed, water deficit appeared to be associated with less symptom expression (Serra et al. 2018). A more recent study confirmed the correlation between rainfall and symptom expression and demonstrated the role played by temperature variation on the annual variability and severity of foliar symptoms (Calzarano et al. 2019). Moreover, they also highlighted age-related differences in this correlation based on 21 consecutive years of observation (Calzarano et al. 2019).

Variations on the incidence of foliar symptoms of ED in different climatic condition have also been investigated. The investigation revealed a positive correlation between symptoms expression and winter rainfall 18 months before and the presence of severe foliar symptoms was correlated with very high and very low precipitation during the early spring (Sosnowski et al. 2007).

For BD, a survey in France showed correlation of water availability and foliar symptoms expression (Larignon et al. 2009). This is the only available field study in the literature for BD, to date. Thus, more comprehensive field studies on how climatic conditions can influence the foliar symptoms associated with BD, including the role of PMs produced *in planta*, could be fundamental for
elucidating the relationship between fungal PMs and physiological changes in the plants resulting in the expression of foliar symptoms.

Another result arising from this study was the negative detection of protocatechuic acid and spencertoxin from vines inoculated with *Do. vidmadera* and *S. viticola*, respectively. These two species were shown to produce these PMs *in vitro* (Reveglia et al. 2018b, Reveglia et al. 2018c). The negative detection of these PMs may be due to different reasons: i) they may not be produced *in planta*; ii) they could be detoxified by the plant; iii) they may form toxin conjugates with other compounds; and/or iv) they may be irreversibly bonded to the wood contributing to lesion expression. The latter hypothesis was already suggested for others phytotoxins produced by the ED pathogen, *E. lata* (Mahoney et al. 2005, Lardner et al. 2006, Rolshausen et al. 2008). Overall, the general conclusion may be that not all the secondary metabolites produced *in vitro* can be detected *in planta* since their fate mainly depends on the biological role played in the interaction with the host.

In conclusion, this multidisciplinary approach was appropriate for gaining insight into the biological role played by *(R)*-mellein in the interaction of the pathogen with the plant and in the expression of BD symptoms. On this basis, future investigations could be oriented to optimising a quantitative LC/MS-MS method for the quantification of *(R)*-mellein and other PMs reported to be involved in BD symptoms like terremutin in symptomatic wood. Investigations on a possible correlation between the amount of PMs and amount of DNA copies of the pathogen could provide important information on the biology of the pathogens and stage of infection and, hopefully, may shed light on the function of *(R)*-mellein in the development and expression of the disease.
Chapter 5 – Investigation on the Genes Involved in (R)-mellein Biosynthesis in Australian *Botryosphaeriaceae* Isolates

5.1 Introduction

Pathogenic fungi have the ability to produce several SMs resulting in a competitive advantage towards other microorganisms. These compounds have a high functional diversity that reflect a wide range of biological functions, indeed they could be involved in the interaction with the host, virulence, biotic or abiotic stress protection and in some cases, they could be involved in symbiotic relationships (Kusari et al. 2012, Nielsen et al. 2017, Ebert et al. 2019). However, it is not easy to correlate a SM to specific functions involved in plant-pathogen interactions such as the infection process and disease symptoms expression. For this reason, multidisciplinary approaches using chemistry techniques to analyse complex organic extracts and molecular biology techniques to identify genes involved in their biosynthesis are necessary to gain deeper knowledge.

To date, only about 25% of the fungal SM gene clusters have been functionally characterised (Pusztahelyi et al. 2015). Each fungus usually has 20-50 SM genes and ecologically relevant SM gene clusters and their production are highly regulated, often in response to specific biotic interactions and environmental perturbations (Pusztahelyi et al. 2015). Pathogenicity genes are defined as genes necessary for disease development and they may be associated with virulence factors and, in particular, with the biosynthetic pathway of phytotoxic metabolites (Idnurm and Howlett 2001, Lim et al. 2012, Morales-Cruz et al. 2015, Massonnet et al. 2018b).

Among the PMs produced by pathogenic fungi, PKs represent a big and diverse family of SMs that are assembled from the condensation of acetate and malonate units by PKs enzymes (Dewick, 2002). Fungi possess different kind of PKs: non-reducing PKs (nrPKS), partially reducing PKs (prPKs) and finally the highly reducing PKs (hrPKs). The prPKs are less widespread and are considered rare (Cox et al., 2018). In addition, the product of a PKs can be further modified by other enzymes to yield the final metabolite (Cox 2007, Ebert et al. 2019). High diversity in the biological activity has been reported in the literature for PKs. Some of these compounds are known to have anticancer, antioxidant, antimicrobial activity and have been used in the development of pharmaceutical drugs (Cox et al., 2018). On the other hand, they are also mycotoxins and are able to
produce phytotoxic effects on important agricultural crops (Cox et al., 2018). Considering their high structural variability and their diverse biological activity, further genetic and biochemical investigations could provide new insights into the function of PKs enzymes in producing a wide range of bioactive natural compounds.

(R)-Mellein and its derivatives belong to the class of polyketide natural compounds, in particular they are 3,4-dihydroisocoumarines. Dihydroisocoumarines are produced by a wide range of fungal species and insects and they exhibit diverse biological activities: fungicidal, antibacterial, phytotoxicity and HCV protease-inhibitory properties (Krohn et al. 1997). Furthermore, important phytoalexins belonging to this family, for instance 6-methoxymellein is the major phytoalexin from carrots, its biosynthetic pathway has been investigated and the polyketide synthase responsible for its production has also been characterised (Kurosaki et al. 1989, 1993 and 2002).

An example of an iterative prPK enzyme involved in the (R)-mellein synthesis was demonstrated in *Saccharopolyspora erythrea* (Sun et al. 2012). To date, there is only one reported sequence of fungal PKs involved in (R)-mellein synthesis. Chooi et al. (2015) studied the gene involved in the production of (R)-mellein by the wheat pathogen, *P. nodorum*. Their results showed that SN477 was the most highly expressed PKs gene *in planta*, and analysis of the DNA sequence indicated that it encodes for typical prPKS and was similar with an identical domain architecture to the prPKS ATX from *Aspergillus terreus*, which synthesizes 6-MSA. The authors further confirmed the results by heterologous expression of SN477 in yeast. The gene knock-out SN477 resulted in a *P. nodorum* mutant that was not capable of producing (R)-mellein as shown by HPLC metabolic profiling. Thus, SN477 is the first fungal prPKS shown to produce a PKs compound other than 6-MSA. However, its biosynthesis was highly parallel to that of 6-MSA but requires additional chain elongation and keto reduction steps (Chooi et al., 2015).

On the other hand, almost all the example of prPKS reported in literature are involved in 6-methylsalicylic acid (6-MSA) biosynthesis and all the 6-MSA synthases (6-MSASs) show high similarity in the amino acid sequence and domain architecture, corroborating the idea that they have a common ancestor (Fujii et al. 1996, Guo et al. 2014). Furthermore, previous investigation demonstrated that fungi acquired the 6-MSA-type prPKSs by horizontal gene transfer from bacteria (Schmitt and Lumbsch 2009).
Whole genome sequencing, comparative genomics, transcriptomics and others genomic analyses have a fundamental role in characterising the gene clusters involved in the synthesis of SMs. Moreover, these methods are useful for studying the phylogenetic relationships among species (Ebert et al. 2019). Differences in the expression of the genes involved in secondary metabolism may result in high differences in virulence and pathogenicity also among the same species (Cacho et al. 2015, Massonnet et al. 2018a, Massonnet et al. 2018b, Wu et al. 2019).

Botryosphaeriaceae species are generally genetically diverse and this may be the reason for their success in adapting to various lifestyle and environmental conditions (Slippers et al. 2007). However, low genetic variability has reported from many geographical locations for some of the species such as Botryosphaeria, Diplodia and Lasiodiplodia (Chethana et al. 2016). Nevertheless, they are highly variable in their pathogenicity and these observations have led to the initiation of further molecular studies to demonstrate correlations between genetic diversity, pathogenicity and virulence (Úrbez-Torres and Gubler 2009, Baskarathevan et al. 2012, Billones-Baaijens et al. 2013, Pitt et al. 2013a).

To date, there are no documented candidate sequences for any of the potential virulence functions associated with the causal pathogens of GTDs. However, a key study on virulence factors of D. seriata and N. parvum showed that many SM gene clusters were mostly associated with the synthesis of PKs and fatty acid-derived compounds, terpenes, non-ribosomal peptides and amino acid derived compounds (Morales-Cruz et al. 2015). Furthermore, in the case of N. parvum, co-expression analysis identified clusters of virulence factors, suggesting a complex, multi-layered regulation of the virulence repertoire of N. parvum (Massonnet et al. 2018b). So far, different phytotoxic PKs have been isolated from pathogens involved in GTDs and their amount and structural variability might reflect the capability of these fungi to colonise the wood and to producing necroses (Ramírez-Suero et al. 2014, Masi et al. 2018).

Australian Botryosphaeriaceae species are capable of producing well-known and novel PMs (Chapters 2 and 3). Preliminary investigations on 24 fungal isolates representing the nine most widespread and most virulent Botryosphaeriaceae species provided evidence that they were capable of producing PMs when grown in vitro. The chromatographic analysis of their organic extracts confirmed that all isolates produced several and different metabolites. The main metabolite was identified as (R)-mellein (Reveglia et al. 2019). Moreover, (R)-mellein was detected in vine wood showing Botryosphaeria dieback symptoms(Abou-Mansour et al. 2015, Chapter 4 of this thesis).
These results suggested that this PM could have a role in the host-pathogen interaction and disease development. The most recent investigation showed that this PM could weaken the plant immune system and promote infection by the pathogen (Trocal-Aziz et al. 2019).

6-MSA and (R)-mellein have been previously identified from the culture filtrates of *N. parvum* (Abou-Mansour et al., 2015). However, there are no proposed DNA sequences involved in the biosynthesis of (R)-mellein in *Botryosphaeriaceae* spp. causing BD. Considering that the 6-MSA biosynthesis is parallel with that of (R)-mellein, identifying the 6-MSAs in Australian isolates of *Botryosphaeriaceae* spp. could be a useful starting point to identify the genes involved in (R)-mellein biosynthesis. Therefore, the main aim of this chapter was to identify the potential biosynthetic pathway for (R)-mellein production, starting from the identification of the gene involved in the biosynthesis of 6-MSA in Australian isolates of *Botryosphaeriaceae*. Firstly, the capability of *D. seriata* H141a and *N. parvum* DAR78998 to produce (R)-mellein *in vitro* was assessed by HPLC quantification. Using gene reference sequences for 6-MSA from GenBank, primers were designed and tested on Australian *Botryosphaeriaceae* species to determine if these sequences are conserved among the members of the *Botryosphaeriaceae*.

### 5.2 Materials and Methods

#### 5.2.1 Fungal isolates and cultural conditions for (R)-mellein production

*D. seriata* (H141a) and *N. parvum* (DAR78998) stored at the Australian Scientific Collections Unit (Orange, NSW, Australia) were used in this study. The isolates were grown under stationary conditions in each flask containing 1 L of modified Czapek Dox–liquid medium with 0.5% yeast and 0.5% malt extract (pH 6.8). The liquid medium was inoculated with 15 mycelial plugs (8 mm) of each isolate grown on PDA for 1 week. The liquid cultures for each isolate were incubated at 25°C in the dark for 14 days after which the mycelial mats were removed by filtration through four layers of filter paper and kept at -20°C until further processing.

#### 5.2.2 Extraction of (R)-mellein from culture filtrates of *D. seriata* H141a and *N. parvum* DAR78998

The 1 L culture filtrates of *D. seriata* H141a and *N. parvum* DAR78998 were reduced to 500 ml using a rotary evaporator. The solutions were exhaustively extracted with EtOAc (3 x 300 ml) and the organic extracts were combined, dried (Na₂SO₄), and evaporated under reduced pressure. A
yellow residue of 103 mg was obtained from the filtrate of *D. seriata* H141a, while a brown residue of 143 mg was obtained from the filtrate of *N. parvum* DAR 78998.

### 5.2.3 HPLC analysis

The HPLC system (HITACHI) consisted of a pump (5160) and a spectrophotometric detector (5410). The HPLC separations were performed using a Phenomenex Luna C-18 reversed-phase column (15 × 4.6 mm i.d.; 5 μm). The mobile phase used to elute the samples was ACN–H₂O at a flow rate of 1 ml min⁻¹. The chromatographic conditions were in accordance with those previously reported (Ramírez-Suero et al. 2014). The analysis was performed using a gradient starting from 5% of ACN linear increased to 70% in 30 min and finally 100% in 35 min. Detection was performed at 320 nm, corresponding to the maximum UV absorption of (R)-mellein. Samples were injected using a 20 μl loop and monitored for 40 min. Construction of the HPLC calibration curve (Table 5.1) for quantitative (R)-mellein determination was performed with absolute amounts of the (R)-mellein standard dissolved in MeOH in the range between 0.001 and 1 mg/ml, in triplicate for each concentration. The retention times were highly reproducible, varying less than 0.4 min. A HPLC linear regression curve (absolute amount against chromatographic peak area) for (R)-mellein was obtained based on weighted values calculated for seven concentrations of the standard in the above range. The presence of (R)-mellein in the extract was confirmed by co-injection with a pure standard. The quantitative determination of the metabolite was calculated by interpolating the mean area of the chromatographic peak using the equation from the calibration curve (Table 5.1).

<table>
<thead>
<tr>
<th>Rt (min)</th>
<th>Range (C mg ml⁻¹)</th>
<th>R²</th>
<th>n of data points</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.650</td>
<td>0.001 – 1</td>
<td>0.9997</td>
<td>21</td>
<td>0.043</td>
</tr>
</tbody>
</table>

*a Calculated in the form y = a + bx, where y is the chromatographic peak area and x is the C (mg ml⁻¹) of (R)-mellein. The calibration curve for this analysis run was: y = 2E+07x + 333265.*

### 5.2.4 DNA extraction from fungal cultures

The DNA samples were extracted from the mycelia of *D. seriata* H141a and *N. parvum* DAR78998 using PrepMan Ultra using the method described in Chapter 4, Section 4.2.5.

### 5.2.5 Primer development
To search for *Botryosphaeriaceae* DNA sequences that may be potentially involved in (R)-Mellein synthesis, the Basic Local Alignment Search Tool (BLASTn) was used with the 5358 bp (R)-mellein synthase gene sequence of *P. nodorum* strain SN 15 (Accession No. KM365454.1; Chooi et al. 2015) as the reference sequence. The search was optimised for more dissimilar sequences (discontiguous megablast) and limited to nucleotide sequences from *Botryosphaeriales* taxid. BLASTn identified *N. parvum* (UCRNP2; Accession No. XM_007585409.1) and *D. corticola* (BKCO1_5600057) as the two most similar sequences at 61% and 59% homology, respectively. These sequences are a putative 6-methylsalicylic acid (6-MSA) synthase protein mRNA.

To investigate whether the 6-MSA gene is conserved among the members of the *Botryosphaeriaceae* family, primers were initially designed by aligning *N. parvum* (UCRNP2; XM_007585409.1) 5340 bp sequences with the sequences of *P. nodorum* strain SN 15 (Accession No. KM365454.1; Chooi et al. 2015). The sequences with ~60% homology were used to design seven primer pairs using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 5.2).

Table 5.2. Primers with the corresponding sequences, product length and melting temperature (Tm) designed to amplify the 6-methylsalicylic acid (6-MSA) synthase protein mRNA using the *N. parvum* (UCRNP2; XM_007585409.1) as a reference sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’- 3’</th>
<th>Product length</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP1</td>
<td>F CATCCACCGACTTCCTGACC</td>
<td>502</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R AGCTTTGGCGTAGAACCACTC</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NP2</td>
<td>F TGATGGCCGTCGAAGTACGC</td>
<td>749</td>
<td>60.1</td>
</tr>
<tr>
<td></td>
<td>R GGTCAGGAAGTCGGTGATG</td>
<td>60.1</td>
<td></td>
</tr>
<tr>
<td>NP3</td>
<td>F CCATCCAGGTCGTACCTAC</td>
<td>629</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R CGTCGACACTTTGGAGAACA</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>NP4</td>
<td>F CCGAAGAAGAGATGGGAGCC</td>
<td>809</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R GCCTCGATGTAGTTGACGCT</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NP5</td>
<td>F CGCAACATCGACTACTGGGT</td>
<td>899</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R TCCTTCTGATGCGTAGTG</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>NP6</td>
<td>F TCAAGACCGACATCGCCTC</td>
<td>773</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R GATGATCTGGACTTCCCGGC</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>NP7</td>
<td>F AGCTCGACGACAAGACGAAG</td>
<td>745</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R AAGCGCATGGACTGGAACCTT</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>
To test the primers, a gradient PCR was performed using a thermal cycler (C100 Thermal Cycler, BIO-RAD Laboratories, Pty, Ltd., USA). The 25 µl PCR reactions contained 1× PCR buffer (Bioline, UK), 0.4 µM of each primer (Geneworks, Pty. Ltd., Australia), 1.25 U of MyTaq DNA Polymerase, and 1 µl of gDNA of *N. parvum* DAR78998 or *D. seriata* H141a. The thermal cycling conditions were: initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s annealing (gradient run of 54°C to 64°C) and 30 s at 72°C, and a final extension of 72°C for 5 min. Following amplification, 5 µl of each PCR product was separated by electrophoresis (10 V/cm for 50 min) in a 1% agarose gel in 1× TAE buffer. Gels were stained with 1× GelRed nucleic acid gel stain and visualised under UV light using a Gel Doc XR+ Imaging System (BIO-RAD Laboratories, Pty, Ltd., USA).

Since initial results showed only one primer pair (NP1F and NP1R) was able to amplify the *N. parvum* DNA and no PCR product was amplified using *D. seriata* DNA, primer development was further attempted by sequencing the *N. parvum* PCR product using the NP1F and NP1R following the methods previously described. The ~500 bp sequence obtained from the NP1F and NP1R PCR product was further used to search for homologous *Botryosphaeriaceae* DNA sequences that may be involved in 6-MSA or *(R)-Mellein* synthesis using BLASTn. The sequences with 70-99% homology (Table 5.3) were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/) and further aligned using Mega7 to identify potential binding sites for a group-specific primer that can amplify 6-MSA gene for *Botryosphaeriaceae* species.

Table 5.3. *Botryosphaeriaceae* DNA sequences retrieved from GenBank (https://www.ncbi.nlm.nih.gov/) with 75-99% homology to *N. parvum* DAR78998 PCR product using NPF1 and NPR1 primers. These reference sequences are putative genes involved in the expression of 6-methylsalicylic acid synthase.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate/Strain</th>
<th>Accession No.</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Neofusicoccum parvum</em></td>
<td>UCRNP2</td>
<td>AORE01001061.1</td>
<td>99.7%</td>
</tr>
<tr>
<td><em>Botryosphaeria dothidea</em></td>
<td>LW030101</td>
<td>MDSR01000050.1</td>
<td>81.5%</td>
</tr>
<tr>
<td><em>Diplodia seriata</em></td>
<td>DS831</td>
<td>LAQI01000017.1</td>
<td>77.8%</td>
</tr>
<tr>
<td><em>Dothiorella corticola</em></td>
<td>BKCO1</td>
<td>XM_020277419.1</td>
<td>77%</td>
</tr>
<tr>
<td><em>Lasiodiplodia theobromae</em></td>
<td>CSS-01s</td>
<td>MDYX01000023.1</td>
<td>75%</td>
</tr>
</tbody>
</table>
5.2.6 Amplification of 6-methylsalicylic acid synthase using group specific primers

The group-specific primers BotMSAF1 and BotMSAR1 were initially tested by gradient PCR using the genomic DNA (gDNA) of *N. parvum* DAR78998 and *D. seriata* H141a to determine the optimum annealing temperature following the methods previously described. PCR was further performed using the gDNA of Australian *Botryosphaeriaceae* (Table 5.4) from the fungal DNA collection at the National Wine and Grape Industry Centre (NWGIC), Charles Sturt University using the optimum annealing temperature and the thermal cycling conditions previously described. These DNA samples were previously identified by DNA sequencing of the ribosomal DNA (Pitt et al. 2010.; Qiu et al. 2010; Wunderlich et al. 2011). Following PCR amplification, 5 µl of each PCR product was separated by electrophoresis following the methods described previously.

Table 5.4. *Botryosphaeriaceae* fungal isolates used for testing the (R)-mellein gene by PCR.

<table>
<thead>
<tr>
<th><em>Botryosphaeriaceae</em> spp.</th>
<th>Isolates/DAR number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Botryosphaeria dothidea</em></td>
<td>DAR79241^a^</td>
<td>Pitt et al. 2010</td>
</tr>
<tr>
<td></td>
<td>DAR79242^a^</td>
<td>Pitt et al. 2010</td>
</tr>
<tr>
<td></td>
<td>DAR79239^a^</td>
<td>Pitt et al. 2010</td>
</tr>
<tr>
<td><em>Lasiodiplodia theobromae</em></td>
<td>WP-C2^b^</td>
<td>Pitt et al. 2010</td>
</tr>
<tr>
<td></td>
<td>DAR81024^a^</td>
<td>Pitt et al. 2010</td>
</tr>
<tr>
<td><em>Diplodia seriata</em></td>
<td>H141a^b^</td>
<td>Qiu et al. 2010</td>
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<td></td>
<td>B135^b^</td>
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<td>DAR79990^a^</td>
<td>Qiu et al. 2010</td>
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<td><em>Diplodia mutila</em></td>
<td>DAR 79137^a^</td>
<td>Pitt et al. 2010</td>
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<td>DAR79135^a^</td>
<td>Pitt et al. 2010</td>
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<td><em>Neofusicoccum australe</em></td>
<td>DAR79505^b^</td>
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<td>DAR79506^a^</td>
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<td>DAR81004^b^</td>
<td>Wunderlich et al. 2011</td>
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<tr>
<td><em>Neofusicoccum luteum</em></td>
<td>DAR81016^a^</td>
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<td>DAR81019^a^</td>
<td>Wunderlich et al. 2011</td>
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<td><em>Neofusicoccum parvum</em></td>
<td>DAR78998^a^</td>
<td>Pitt et al. 2010</td>
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<td></td>
<td>E12b^b^</td>
<td>Qiu et al. 2011</td>
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<tr>
<td><em>Dothiorella vidmadera</em></td>
<td>DAR78994^a^</td>
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<td>DAR78993^a^</td>
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<td>DAR78992^a^</td>
<td>Pitt et al. 2013</td>
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<td><em>Spencermartinsia viticola</em></td>
<td>DAR78870^a^</td>
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<td>DAR78869^a^</td>
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<td></td>
<td>DAR78867^a^</td>
<td>Pitt et al. 2010</td>
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^a Agricultural Scientific Collections Unit, NSW DPI, Orange, Australia.
^b National Wine and Grape Industry, Charles Sturt University, Wagga Wagga, NSW, Australia.
5.2.7 Phylogenetic analysis

All PCR products of the seven Botryosphaeriaceae species that were amplified using the group-specific primers BotMSAF1 and BotMSAR1 were sent for further two-way DNA sequencing at the AGRF following the protocol previously described in section 4.2.5. The resulting sequences (Appendix D) and chromatographs were analysed using DNAMAN 5.2 (Lynnon Biosoft) and Chromas Ver 2.6.4 (Technelysium Pty Ltd, Australia). All sequences were aligned with the reference sequences retrieved from GenBank (Table 5.3) using MEGA7 (Tamura et al. 2007). The (R)-mellein synthase gene sequence of P. nodorum strain SN15 (Accession No. KM365454.1) was used as the outgroup. The aligned sequences were tested for phylogeny and a neighbour joining tree was generated. All the DNA sequences are reported in the Appendix C.

5.3 Results

5.3.1 HPLC analysis for the production of (R)-mellein in vitro by D. seriata H141a and N. parvum DAR78998

The HPLC chromatograms of D. seriata and N. parvum are shown in Figure 5.1 and 5.2. The metabolic profiles of the two species were qualitatively similar in both chromatograms indicating the (R)-mellein peaked at 21.67 min.

However, the quantification of (R)-mellein indicated that N. parvum DAR78998 produced 0.29 ± 0.01 mg ml\(^{-1}\), while D. seriata H141a produced a lower amount of (R)-mellein at 0.10 ±0.01 mg ml\(^{-1}\).
5.3.2 Amplification of 6-methyl salicylic acid (6-MSA) synthase gene for *Botryosphaeriaceae* spp

Of the seven primer pairs initially designed, only NPF1 and NPR1 were able to amplify a 500 bp DNA fragment from *N. parvum* DAR78998, while none of the other tested primers were able to amplify *D. seriata* H141a gDNA. The BLASTn analysis for the resulting sequence showed similarity with the putative gene involved in the expression of the 6-MSA synthase protein in *N. parvum* (99%) and *D. corticola* (78%). The BLASTn search that was widened to whole genome sequences further revealed that the *N. parvum* DAR78998 500 bp sequences shared 75 to 99.7%
homology with *Botryosphaeriaceae* DNA sequences from GenBank (Table 5.3). These homologous sequences are putative genes involved in the expression of 6-MSA.

The alignment of the reference *Botryosphaeriaceae* DNA sequences (Table 5.4) identified a binding site suitable for group-specific primers. The primers BotMSAF1 (5’-CAAGGGCATCACCAGCAT-3’) and BotMSAR1 (5’-CSGTCATSACGCTGCTCSA-3’) which was degenerate at nucleotide 2, 8 and 17 were designed to anneal to the 6-MSA gene of *N. parvum*, *D. seriata* and *L. theobromae*. Preliminary tests using gradient PCR showed the optimum annealing temperature for BotMSAF1 and BotMSAR1 was 64°C which amplified a single 380 bp PCR product for *N. parvum* DAR78998 and *D. seriata* H141a (Figure 5.3) while lower temperatures resulted in non-specific amplifications for *D. seriata* gDNA (Figure 5.3).

![Figure 5.3. Gel electrophoresis of gradient PCR to determine the optimum annealing temperature for *Botryosphaeriaceae* group-specific primers BotMSAF1 and BotMSAR1. The numbers on the top of each band denote the annealing temperatures, the number on the far left denote the molecular weight of the 50 bp DNA ladder. Lanes 1-8 *D. seriata* H141a; lane 9 non-template control (NTC); lanes 10-17 *N. parvum* DAR 78998.](image)

The PCR analysis of other *Botryosphaeriaceae* DNA (Table 5.4) further showed the primers BotMSAF1 and BotMSAR1 were able to amplify all representative DNA samples for *N. australe*, *N. luteum*, *N. parvum*, *L. theobromae* and *Do. vidmadera*, while none of the *B. dothidea* and *S. viticola* gDNA samples were amplified (Figure 5.4).
Figure 5.4. Gel electrophoresis for the PCR using BotMSAF1 and BotMSAR1 primers. The numbers on the far left denote the molecular weight of the 50 bp DNA ladder. a) Lanes 1-3 *D. seriata* DAR79990, B135, H141a; lanes 4-5 *D. mutila* DAR 79137, DAR79135; lane 6 negative control *E. lata* WB8; lane 7 non-template control NTC; lanes 8-10 *N. austral* DAR79505, DAR 79506, DAR81004; lanes 11-12 *N. luteum* DAR81016, DAR81019; lanes 13-14 *N. parvum* E12b, DAR78998. b) Lanes 1-3 *B. dothidea* DAR79241, DAR79242, DAR79239; lanes 4-5 *L. theobromae* WPC2, DAR81024; lanes 6-8 *Do. vidmadera* DAR78994,78992,78993; lanes 9-11 *S. viticola* DAR78867, DAR78869, DAR78870.

### 5.3.3 Phylogenetic analysis

The neighbour joining tree generated from the aligned sequences of representative isolates for *Botryosphaeriaceae* species from Australia and their homologues from the GenBank database are presented in Figure 5.5. The neighbour joining tree had two major branches with one branch further divided into sub-branches (Figure 5.5). The first branch was occupied by *D. mutila* while the second branch was further divided into five branches with one cluster for *N. austral* and *N. luteum* and separate clusters for *N. parvum*, *Do. vidmadera*, *L. theobromae* and *D. seriata*. The reference sequences for Australian isolates of *D. seriata*, *L. theobromae* and *N. parvum* clustered in the same
branch with their matching accessions from GenBank while the reference gene accession for *D. corticola* clustered on the same branch with its close relative, *D. seriata*.

Figure 5.5. Neighbour joining tree obtained from the 6-MSA synthase gene sequence data of representative Botryosphaeriaceae isolates from Australia and the representative sequences from the GenBank database (indicated by red dots). The red dotted line indicates the main branches of the tree. The tree is rooted to the outgroup *Parastagonospora nodorum*.

5.4 Discussion

The main objective of this work was to investigate the possible gene sequences involved in the biosynthesis of (R)-mellein in Australian isolates of Botryosphaeriaceae using the gene related to 6-MSA synthesis as a starting point. Both 6-MSA and (R)-mellein are synthesised by prPKs, that is considered a rare PK in fungi compared to the nrPKS and hrPKs (Cox et al., 2018). Chun-jun Guo et al. (2014) showed that PKs including terreic acid and patulin produced by *Aspergillus therreus* and *Penicillium expansum*, respectively, are compounds derived from 6-MSA. The phylogenetic
analysis performed in this study showed that the \((R)\)-mellein gene sequences from \textit{P. nodorum} (Chooi et al., 2015) had some sequence homology with the 6-MSA synthase gene found in certain \textit{Botryosphaeriaceae} species. This led to the investigation to assess the possible link between \((R)\)-mellein synthesis and the presence of 6-MSA genes in the genome of \textit{Botryosphaeriaceae} species. This work is a first step in elucidating the pathway for the synthesis of \((R)\)-mellein produced by the \textit{Botryosphaeriaceae}.

Pathogenic fungi belonging to the \textit{Botryosphaeriaceae} and involved in BD are known to produce bioactive isocoumarines that could have a role in pathogenicity and virulence (Masi et al., 2018). However, prior to the investigation reported in Chapter 2, there was a lack of information on the production of PMs from Australian \textit{Botryosphaeriaceae} isolates. The preliminary analysis of the culture filtrates of nine \textit{Botryosphaeriaceae} spp. resulted in the identification of \((R)\)-mellein from the organic extracts of \textit{D. mutila}, \textit{D. seriata}, \textit{N. australe} and \textit{N. luteum} (Reveglia et al., 2019). The HPLC quantification of this PM in the organic extract of selected isolates of \textit{D. seriata} and \textit{N. parvum} reported in this chapter also confirms that Australian \textit{Botryosphaeriaceae} species can produce \((R)\)-mellein. Furthermore, the higher concentration of \((R)\)-mellein in the organic extract of \textit{N. parvum} confirmed that this species can produce higher amounts of this PM compared to \textit{D. seriata} as reported by Ramirez-Suero et al. (2014).

Considering the lack of reference sequences involved in \((R)\)-mellein biosynthesis in \textit{Botryosphaeriaceae} available to date, this study used the gene sequence reported for its biosynthesis in \textit{P. nodorum} (Chooi et al. 2015), as the reference in the discontiguous megablast research limited to nucleotide sequences from \textit{Botryosphaeriales} taxid. As a result, two closely related putative 6-MSA synthase protein mRNA sequences were identified for \textit{N. parvum} (UCRNP2; Accession No. XM_007585409.1) and \textit{D. corticola} (BKCO1_5600057). Seven primer pairs were initially designed using the \textit{N. parvum} sequence retrieved from GenBank as a reference. Of the seven primers tested, only NPF1 and NPR1 successfully amplified \textit{N. parvum} DNA but were not able to amplify \textit{D. seriata} DNA. Further attempts to design primers using the \textit{N. parvum} sequences identified a binding site suitable for group-specific primers. The primers BotMSAF1 and BotMSAR1 were able to amplify sequence from isolates of \textit{D. seriata}, \textit{D. mutila}, \textit{Do. vidmadera}, \textit{L. theobromae}, \textit{N. australe}, \textit{N. luteum} and \textit{N. parvum} but not for \textit{B. dothidea} and \textit{S. viticola} (Reveglia et al. 2019).
Since HPLC analysis showed that Australian isolates of *D. seriata* and *N. parvum* produced (R)-mellein and other closely related isocoumarines *in vitro* and *in planta* (Reveglia et al. 2019, Chapter 4), it is possible that the DNA amplified from representative *Botryosphaeraceae* species could be involved in the biosynthesis of (R)-mellein via the 6-MSA pathway.

Previous investigations for PMs of *Do. vidmadera* resulted in the isolation and identification of six polyphenols and (R)-mellein or 6-MSA were not detected by TLC nor isolated from the culture filtrates (Reveglia et al 2018c). However, the Australian isolates of this species were positive to the PCR using BotMSAF1 and BotMSAR1 primers. A possible explanation could be that the genes that were amplified by PCR may be involved in the biosynthesis of another PKs, including the isolated polyphenols, with a biosynthesis close to that of 6-MSA. Polyphenols derived from directed cyclocondensations of poly-β-keto intermediates or from partially reduced PK chains produced by iterative PKSs can also utilise a non-acetate starter (Hertweck, 2009). These enzymes can generate and stabilise the highly reactive polyphenols intermediates through pre-organisation of their folding mode (Hertweck, 2009). Most fungal polyphenols result from enzymes with an F-type folding (Hertweck, 2009).

The *L. theobromae* isolates were positive in the PCR analysis. These species were not included in the preliminary investigation on the production of PMs (Reveglia et al. 2019). However, this species has been reported to produce isocoumarines including (R)-mellein and other PKs (Aldridge et al., 1971).

The isolates of *B. dothidea* and *S. viticola* were negative in the PCR analysis. To our knowledge, *B. dothidea* has never been reported to produce 6-MSA or (R)-mellein, while PMs from *S. viticola* were studied for the first time during this PhD project and among the PMs identified, none of the target PKs were isolated (Reveglia et al., 2018b). Overall, these results suggest that these genes are likely to be involved in the production of 6-MSA or (R)-mellein and are not conserved in these two *Botryosphaeriaceae* species.

It is important to note that this study is preliminary and further investigations are required to identify the genes involved in the biosynthesis of (R)-mellein in *Botryosphaeriaceae*. The sequences obtained from the amplification *Botryosphaeriaceae* DNA using BotMSAF1 and BotMSAR1 (Appendix D) should be further studied to confirm that they are indeed involved in the biosynthesis of (R)-mellein, 6-MSA or other related PKs. For instance, heterologous gene
expression followed by metabolic profiling could assist in the identification of the synthesised PK. Moreover, gene knockout experiments could generate a series of mutants that could be used for \textit{in vitro} production of \((R)\)-mellein and PKs followed by pathogenicity studies. The isolation of the PKs from mutant strains combined with bioinformatics analysis may allow the proposition of the biosynthetic pathway for \((R)\)-mellein production. These pathogenicity studies will provide further insight into the role of PMs in the pathogenicity and virulence of \textit{Botryosphaeriaceae} spp. involved in BD.
Chapter 6 – Overall Conclusion and Future Perspectives

The objective of this PhD project was to investigate the production of PMs produced by the most virulent and widespread isolates of *Botryosphaeriaceae* spp. in Australian vineyards. It is important to highlight, that prior to this investigation, no data was available in literature on the PMs associated with Australian isolates of the *Botryosphaeriaceae*. Moreover, the role of target metabolites *in planta* and the genes involved in 6-MSA and (R)-mellein biosynthesis were also investigated. In this chapter, the main conclusions arising from the experimental work are summarised and potential areas of further research are outlined. Insights from this research will provide knowledge on the role of PMs as possible virulence factors and may provide future possibilities for selecting biological marker(s) for the early detection of the causal agents of BD and other GTDs. The main aims of this research were to: (i) isolate, biologically and chemically characterise the PMs produced by different species of *Botryosphaeriaceae* from Australian vineyards; (ii) investigate the production of PMs *in planta* and to develop a LC/MS-MS protocol to analyse naturally infected and artificially infected vines; and (iii) investigate the genetic mechanisms surrounding the production of phytotoxins.

The data generated from these studies showed that the phytotoxicity of the culture filtrates and organic extracts differed between species and among isolates of the same species. PMs produced by *D. seriata* H141a, *D. mutila* DAR79137, *N. australe* DAR79506, *N. parvum* DAR80004, and for the first time, those produced by *N. luteum* DAR81016, *S. viticola* DAR78870 and *Do. vidmadera* DAR78993 were isolated and fully characterised by spectroscopic techniques (essentially 1D and 2D $^1$H and $^{13}$C NMR and HR ESIMS). The isolated PMs belong to different classes of natural occurring compounds: organic acids, phenolic compounds, quinones, anthraquinones, cyclopentanones and isocoumarines.

Isocoumarines such as (R)- mellein and phenolic compounds have also been isolated from non-Australian *Botryosphaeriaceae* isolates and they are considered typical PMs produced by the *Botryosphaericeae*. PMs from Australian isolates of *S. viticola* and *Do. vidmadera* are also reported in this thesis, no data are available for non-Australian isolates of these species. Data on PMs from non-Australian isolates of *D. mutila* are available in the literature, however investigations on Australian isolates resulted in the characterisation of two novel quinones.
The assignment of the chemical structure to a fungal phytotoxin is important to understand its mode of action and its function in the plant-pathogen interactions. This in turn could assist in developing more specific and effective control methods for plant diseases. The more information we know about the secondary metabolism of a pathogen, the more we can know about its life cycle. Differences in the phytotoxicity of the isolated compounds indicate that not all the SMs produced by a fungal pathogen play a role in the development of disease symptoms. However, they may still play a significant role in the plant-pathogen interaction. Future investigation on how these compounds interact with host cells will assist in understanding their mode of action.

This study further showed that the EPSs produced by three *Neofusicoccum* spp. were phytotoxic at different concentrations on grapevine leaves, and the results agree with those reported in the literature (Andolfi et al. 2009, Cimmino et al. 2016) that these high molecular weight phytotoxins are involved in the virulence of these pathogens, probably synergistically with PMs. Their structural characterisation should be the main goal of future investigations. The PMs should be purified, characterised and assayed in comparison to EPSs from other sources (commercially available or produced by other fungal species) as controls. Furthermore, considering that foliar symptoms have never been observed in BD infected vines in Australian vineyards, it may be useful to perform the bioassays on different grapevine tissues. For instance, an *in vitro* grapevine calli assay, as already reported for testing other phytotoxic compounds (Ramirez-Suero et al. 2014), could be a useful starting point to gain additional general information on the phytotoxic effects of the EPSs on grapevine cells.

Bioactive metabolites from fungi can show a wide range biological activities (Evidente et al. 2014, Cimmino et al. 2015, Masi et al. 2018). For this reason, it could be valuable to assay other biological activities of the isolated compounds. For instance, diploquinone A and B and neoanthraquinone could be screened for different activities (antibiotic, antimicrobial, anticancer etc.) because it is well known that compounds with a quinone and anthraquinone backbone show a wide range of biological activities (Medentsev and Akimenko 1998, Locatelli et al. 2011, Gessler et al. 2013). Finally, data arising from this research could be useful in developing a database of all compounds produced by the corresponding species. This database could assist future investigations for identifying potential bio-marker(s) of BD.
To investigate the role of the PMs in the pathogenicity and virulence of the *Botryosphaeriaceae* pathogens, an *in planta* multidisciplinary experiment using molecular and analytical chemistry techniques was designed. Wood samples symptomatic and asymptomatic for BD were analysed by cultural isolations, qPCR and LC-MS/MS, in particular a LC-QqQ in MRM mode was used. Three target PMs from the *in vitro* studies were selected for the LC-MS/MS: \( (R) \)-mellein, protocatechuic alcohol and spencertoxin. \( (R) \)-mellein was only detected in symptomatic wood samples and none of the target compounds were detected in asymptomatic wood samples. Moreover, the amount of \( (R) \)-mellein detected by LC-MS/MS was correlated with the amount of pathogen DNA detected by qPCR. The detection of \( (R) \)-mellein in infected grapevine wood showing BD symptoms concurs with that previously reported by Abou-Mansour et al. (2015). However, the inability to detect the other two target compounds suggests that the PMs may have a different fate *in planta*, and this may be due to their different biological roles in disease development, fungal life cycle and plant-pathogen interactions. The application of this optimised procedure to other PMs could assist in selecting a suitable pool of biological markers for the early detection of the disease.

The LC/MS-MS method developed in this research in combination with qPCR analysis gave a more comprehensive picture of the infection status of the plant. Multi-species primers targeting *Botryosphaeriaceae* species have been developed for environmental samples including grapevine wood (Ridgway et al. 2011; Spagnolo et al. 2011). However, these assays were designed primarily for conventional PCR and are semi-quantitative. Recently, Pouzolet et al. (2017) developed a qPCR assay, however, it only targets *D. seriata* and its cryptic species. The qPCR protocols using multi-species primers developed by Billones-Baaijens (2018) and used in this study were therefore more reliable for detecting and quantifying a wide range of *Botryosphaeriaceae* species in artificially-inoculated and naturally-infected vines.

Another important finding from this investigation was the absence of \( (R) \)-mellein in non-necrotic wood from an infected vine. This suggests that this PM was not translocated throughout the wood which is the general hypothesis for PMs involved in GTDs (Tey-Rulh et al. 1991). These data highlight that the mere presence of PMs in the wood may not be enough to result in foliar symptoms on grapevines with BD. The development of symptoms could be more complex than previously thought and they could arise from the interaction between biotic and abiotic stress (water stress, drought, heat stress etc). Different stress factors can weaken the plant and therefore result in the development of foliar symptoms. Investigations on the relationship between foliar symptoms
and climatic conditions has been previously reported (Sosnowski et al. 2007, Calzarano et al. 2019, Songy et al. 2019). However, only very limited information is available for BD (Larignon et al. 2009) thus, there is a need for future work to improve the knowledge on the role of PMs in the development of foliar symptoms. Finally, it is very likely that a diseased grapevine shows differences in its metabolome compared to a healthy grapevine. Thus, metabolomic studies might disclose important insights on what happens to a grapevine when it is exposed to different abiotic and biotic stress conditions. Metabolomics is the latest of the omics sciences and its relevance in plant science has increased in the last decades (Fiehn 2002, Schauer and Fernie 2006, Vasilev et al. 2016, Tugizimana et al. 2018). Investigations using complex mixtures, such as wood extracts obtained under different stress conditions, could result in the identification of unique chemical fingerprints that may be related to specific cellular processes, such as defense mechanisms or stress responses of the plant. The analytical chemistry experiments conducted in this study have all the characteristics for being suitable for metabolomics research. Thus, the LC-MS/MS data collected from this work could be used for metabolomics analyses that may shed light on the metabolic changes that occur in a grapevine infected by Botryosphaeriaceae pathogens.

Overall, the data collected during this PhD project, together with data already reported (Djoukeng et al. 2009, Evidente et al. 2010, Ramirez-Suero et al. 2014, Abou-Mansour et al. 2015, Trotel-Aziz et al. 2019) indicates that (R)-mellein is an important PMs produced by Botryosphaeriaceae spp. and that it may play an important role in pathogenicity and virulence. Nevertheless, no information on the genes involved in the biosynthesis of (R)-mellein by Botryosphaeriaceae spp. was available in GenBank. (R)-mellein is a polyketide with a parallel biosynthesis to 6-MSA. This study attempted to investigate the genetic mechanisms surrounding 6-MSA and (R)-mellein production using (R)-mellein gene sequences from another fungal pathogen, P. nodorum, as a reference. Preliminary results indicate that the 6-MSA synthase gene is conserved in some of the Botryosphaeriaceae species. Since this gene shared some level of homology with the (R)-mellein reported in P. nodorum, it is possible that this 6-MSA synthase is a precursor for the biosynthesis of (R)-mellein. However, this work requires further investigation and a heterologous expression system may be useful to demonstrate if the characterised sequence is sufficient to encode for a PR-PKS involved in (R)-mellein production or other closely related polyketides. A gene knockout study could also be used to inactivate the target gene and to stop the production of (R)-mellein. Likewise, the comparison of the virulence of wild type and the mutant of the fungal species could assist in determining if (R)-mellein has any role in the virulence of the pathogen. Transcriptional analysis in planta and in vitro will define the expression dynamics of the proposed gene and may assist in
understanding its role during the interaction with the host and if it is upregulated during the infection processes. Investigations on the possible virulence factors at a genetic level will provide a more comprehensive understanding of the pathogenic mechanisms of the *Botryosphaeriaceae* and comparing this data with those available for the other GTD pathogens will allow understanding of their differences in colonisation strategies, resulting in more accurate diagnostic tools and more specific control methods.

This multi-disciplinary research has demonstrated that *Botryosphaeriaceae* pathogens are capable of producing PMs *in vitro* and *in planta*. This information may contribute to the global understanding of the pathogen life cycle and disease development in vineyards, promoting the development of more specific and environmentally friendly control method for BD.
Chapter 7– References


Reveglia, P., S. Savocchia, R. Billones-Baaijens, M. Masi and A. Evidente (2018b). Spencertoxin and spencer acid, new phytotoxic derivatives of diacrylic acid and dipyridinbutan-1, 4-diol produced by Spencermartinsia viticola, a causal agent of


Appendix A – Presentation and Awards

A.1 Presentations

- **11th IWGTD**, Penticton (CA) 7-12 July 2019. Presentation Title: Investigating the production and translocation of phytotoxic metabolites by Australian *Botryosphaeriaceae* spp. in artificially-inoculated and naturally-infected vines.

- **3rd MS-WineDay**, San Michele dell’Adige (IT) 16-17 May 2019. Presentation Title: Investigating the production and translocation of phytotoxic metabolites by Australian *Botryosphaeriaceae* spp. in artificially-inoculated and naturally-infected vines.

- **CRUSH 2018**: The grape and wine symposium, Adelaide (AU) 24-26 September 2018. Presentation Title: Secondary phytotoxic metabolites produced by species of *Botryosphaeriaceae* implicated in grapevine trunk diseases in Australia.

A.2 Posters

- **11th IWGTD**, Penticton (CA) 7-12 July 2019. Presentation Title: Isolation and characterisation of phytotoxins produced by the *Botryosphaeriaceae* and their role in grapevine trunk diseases.

- **12th SISOC**, Ferrara (IT) 2-4 July 2018. Poster Title: Phytotoxic secondary metabolites produced by species of *Botryosphaeriaceae* involved in grapevine trunk diseases in Australia.


- **10th IWGTD**, Reims (FR) 4-7 July 2017. Poster Title: Characterisation of secondary metabolites produced by Australian species of *Botryosphaeriaceae* involved in grapevine trunk diseases.

A.3 Awards

- **First Place Student Poster Presentation Award**. Presentation Title: Isolation and characterisation of phytotoxins produced by the *Botryosphaeriaceae* and their role in grapevine trunk diseases. **11th IWGTD**, Penticton (CA) 7-12 July 2019.
Appendix B – NMR Spectra

B.1 1D and 2D 1H-NMR and 13C-NMR spectra of novel PMs isolated from Australian Botryosphaeriaceae spp.

Spectra 1. 1H NMR spectrum of spencertoxin (MeOD, 400 MHz).

Spectra 2. 13C NMR spectrum of spencertoxin (MeOD, 100 MHz).

Spectra 3. COSY spectrum of spencertoxin (MeOD, 400 MHz).

Spectra 4. HSQC spectrum of spencertoxin (MeOD, 400/100 MHz).

Spectra 5. HMBC spectrum of spencertoxin (MeOD, 400/100 MHz).

Spectra 6. 1H NMR spectrum of spencer acid (MeOD, 500 MHz).

Spectra 7. 13C NMR spectrum of spencer acid (MeOD, 100 MHz).

Spectra 8. COSY spectrum of spencer acid (MeOD, 500 MHz).

Spectra 9. HSQC spectrum of spencer acid (MeOD, 500/125 MHz).

Spectra 10. HMBC spectrum of spencer acid (MeOD, 500/125 MHz).

Spectra 11. 1H NMR spectrum of 5-hydroxymethy-2-isopropoxyphenol (MeOD, 400 MHz).

Spectra 12. 13C NMR spectrum of 5-hydroxymethy-2-isopropoxyphenol (MeOD, 100 MHz).

Spectra 13. COSY spectrum of 5-hydroxymethy-2-isopropoxyphenol (MeOD, 400 MHz).

Spectra 14. HSQC spectrum of 5-hydroxymethy-2-isopropoxyphenol (MeOD, 400/100 MHz).

Spectra 15. HMBC spectrum of 5-hydroxymethy-2-isopropoxyphenol (MeOD, 400/100 MHz).

Spectra 16. 1H NMR spectrum of diploquinone A (CDCl3, 400 MHz).

Spectra 17. 13C NMR spectrum of diploquinone A (CDCl3, 400 MHz).

Spectra 18. HSQC spectrum of diploquinone A (CDCl3, 400 MHz).

Spectra 19. HMBC spectrum of diploquinone A (CDCl3, 400 MHz).

Spectra 20. 1H NMR spectrum of diploquinone B (CDCl3, 400 MHz).
Spectra 21. $^{13}$C NMR spectrum of diploquinone B (CDCl$_3$, 400 MHz).

Spectra 22. HSQC spectrum of diploquinone B (CDCl$_3$, 400 MHz).

Spectra 23. HMBC spectrum of diploquinone B (CDCl$_3$, 400 MHz).

Spectra 24. $^1$H NMR spectrum of neoanthraquinone (CDCl$_3$, 400 MHz).

Spectra 25. $^{13}$C NMR spectrum of neoanthraquinone (CDCl$_3$, 400 MHz).

Spectra 26. HSQC spectrum of neoanthraquinone (CDCl$_3$, 400 MHz).

Spectra 27. HMBC spectrum of neoanthraquinone (CDCl$_3$, 400 MHz).
Spectra 1. $^1$H NMR spectrum of spencertoxin (MeOD, 400 MHz).
Spectra 2. $^{13}$C NMR spectrum of spencertoxin (MeOD, 400 MHz).
Spectra 3. COSY spectrum of spencertoxin (MeOD, 100 MHz).
Spectra 4. HSQC spectrum of spencertoxin (MeOD, 400/100 MHz).
**Spectra 5.** HMBC spectrum of spencertoxin (MeOD, 400/100 MHz).
Spectra 6. $^1$H NMR spectrum of spencer acid (MeOD, 500 MHz).
Spectra 7. $^{13}$C NMR spectrum of spencer acid (MeOD, 125 MHz).
Spectra 8. COSY spectrum of spencer acid (MeOD, 400 MHz).
Spectra 9. HSQC spectrum of spencer acid (MeOD, 500/125 MHz).
**Spectra 10.** HMBC spectrum of spencer acid (MeOD, 500/125 MHz).
Spectra 11. $^1$H NMR spectrum of 5-hydroxymethyl-2-isopropoxyphenol (CDCl$_3$, 400 MHz).
**Spectra 12.** $^{13}$C NMR spectrum of 5-hydroxymethy-2-isopropoxyphenol (CDCl$_3$, 400 MHz).
Spectra 13. COSY spectrum of 5-hydroxymethyl-2-isopropoxyphenol (CDCl₃, 400 MHz).
Spectra 14. HSQC spectrum of 5-hydroxymethy-2-isopropoxyphenol (CDCl3, 400 MHz).
Spectra 15. HMBC spectrum of 5-hydroxymethy-2-isopropoxyphenol (CDCl3, 400 MHz).
Spectra 16. $^1$H NMR spectrum of diploquinone A (CDCl$_3$, 400 MHz).
Spectra 17. $^{13}$C NMR spectrum of diploquinone A (CDCl₃, 400 MHz).
Spectra 18. HSQC spectrum of diploquinone A (CDCl₃, 400 MHz).
Spectra 19. HMBC spectrum of diploquinone A (CDCl₃, 400 MHz).
Spectra 20. $^1$H NMR spectrum of diploquinone B (CDCl$_3$, 400 MHz).
Spectra 21. $^{13}$C NMR spectrum of diploquinone B (CDCl$_3$, 400 MHz).
Spectra 22. HSQC spectrum of diploquinone B (CDCl₃, 400 MHz).
Spectra 23. HMBC spectrum of diploquinone B (CDCl$_3$, 400 MHz).
Spectra 24. $^1$H NMR spectrum Neoanthraquinone (CDCl$_3$, 400 MHz).
Spectra 25. $^{13}$C NMR spectrum of Neoanthraquinone (CDCl$_3$, 400 MHz).
Spectra 26. HSQC spectrum of Neoanthraquinone (CDCl₃, 400 MHz).
Spectra 27. HMBC spectrum of Neoanthraquinone (CDCl$_3$, 400 MHz).
B.2 $^1$H NMR of already know PMs isolated from Australian Botryosphaeriacae spp.

Spectra 28. $^1$H NMR spectrum of (R)-mellein (CDCl$_3$, 400 MHz).

Spectra 29. $^1$H NMR spectrum of $^1$H NMR spectrum of (3R,4R)-4-hydroxymelleins (CDCl$_3$, 400 MHz).

Spectra 30. $^1$H NMR spectrum of $^1$H NMR spectrum of (3R,4S)-4-hydroxymelleins (CDCl$_3$, 400 MHz).

Spectra 31. $^1$H NMR spectrum of $^1$H NMR spectrum of tyrosol (CDCl$_3$, 400 MHz).

Spectra 32. $^1$H NMR spectrum of p-hydroxybenzaldehyde (CDCl$_3$, 400 MHz).

Spectra 33. $^1$H NMR spectrum of 2-(4-hydroxyphenyl)acetic acid (CDCl$_3$, 400 MHz).

Spectra 34. $^1$H NMR spectrum of benzene-1,2,4-triol (CDCl$_3$, 400 MHz).

Spectra 35. $^1$H NMR spectrum of resorcinol (CDCl$_3$, 400 MHz).

Spectra 36. $^1$H NMR spectrum of 3-(hydroxymethy)phenol (CDCl$_3$, 400 MHz).

Spectra 37. $^1$H NMR spectrum of protocatechuic Alcohol (CD$_3$OD, 400 MHz).

Spectra 38. $^1$H NMR spectrum of nigrosporione (CDCl$_3$, 400 MHz).
Spectra 28. $^1$H NMR spectrum of (R)-mellein (CDCl$_3$, 400 MHz).
Spectra 29. $^1$H NMR spectrum of (3$R$,4$R$)-4-hydroxymellein (CDCl$_3$, 400 MHz).
Spectra 30. $^1$H NMR spectrum of (3R,4S)-4-hydroxymellein (CDCl$_3$, 400 MHz).
Spectra 31. $^1$H NMR spectrum of tyrosol (CDCl$_3$, 400 MHz).
Spectra 32. $^1$H NMR spectrum of p-hydroxybenzaldehyde (CDCl$_3$, 400 MHz).
Spectra 33. $^1$H NMR spectrum of 2-(4-hydroxyphenyl)acetic acid (CDCl$_3$, 400 MHz).
Spectra 34. $^1$H NMR spectrum of benzene-1,2,4-triol (CDCl$_3$, 400 MHz).
Spectra 35. $^1$H NMR spectrum of resorcinol (CDCl$_3$, 400 MHz).
Spectra 36. $^1$H NMR spectrum of 3-(hydroxymethy)phenol (CDCl$_3$, 400 MHz).
Spectra 37. $^1$H NMR spectrum of protocatechuic alcohol (CD$_3$OD, 400 MHz).
Spectra 38. $^1$H NMR spectrum of nigrosporione (CDCl$_3$, 400 MHz).
Appendix C – Statistical Analysis

C.1 ANOVA of lesions caused by *Botryosphaeriaceae* spp. inoculated on grapevines (cv. Chardonnay and Cabernet Sauvignon) 6 months post inoculation.

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C.2 ANOVA of lesions caused by *Botryosphaeriaceae* spp. inoculated on grapevine (cv. chardonnay and cabernet sauvignon) 12 months post inoculation.

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<th>Mean Square</th>
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C.3 ANOVA of qPCR analysis of grapevines wood (cv. chardonnay and cabernet sauvignon) inoculated with *Botryosphaeriaceae* spp. 6 months post inoculation.

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C.4 ANOVA of qPCR analysis of grapevines wood (cv. chardonnay and cabernet sauvignon) inoculated with *Botryosphaeriaceae* spp. 6 months post inoculation.

<table>
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Appendix D- DNA Sequences

D.1 DNA sequences of Australian isolates of *D. mutila*, *D. seriata*, *Do. vidmadera*, *L. theobromae*, *N. australe*, *N. Luteum* and *N. parvum* with high homology with reported gene sequences encoding for 6-MSA biosynthesis in *Botryosphaeriaceae* spp.

*D. seriata* DAR79990

1  CCCTGGGAGC  ACCTGGACAAA  GTTCGACGTG  GCCCCACACG  TCCTAGCGCC  GGGCCGGCC
61  CTCGAGCCCG  GCAAGGGGCGG  GCCGGATTTG  GTCCGAGG  AGATTGCGCC  GGGCGAGTA
121  TCCACACACG  GCGGGGCACG  TCCTAGCGCC  GCTGCCGGCC  AGGGATCAA  GAAGGTCTGC
181  GAAAGGGGCG  CCAGAGCAGC  CCCCCCGCATG  AGCTGCTGAC  TACGCCGAG  GATCGCCGG
241  TGCATCGCGG  CGGATGCTGT  GATGAACGAC  ATCGACGACA  TCGACCACG  CGCGCCGGT
301  G

*D. seriata* B135

1  ACGAGGGTCT  CCCGGCGTGG  GAGCACTGC  CAGCAAGGTG  CAGCTGGCTCC  ACAGCGTCTG
61  CACGCGCGGC  CGGGCTGGAG  AGGCCCAGCA  GGGCCGGCC  ATGCCCGTGG  TCCAGGGAGT
121  TGCGCCGGTG  CGAGATTTCA  CATCGCCGCG  CGCTTCGCTA  GGGCTGCTG  CGGGCCAGGA
181  GTGCAAGAAG  GATGGCTGAG  CGGGCCGAAA  GAGCGCCCGC  AAGCTGAAGG  CGTGCGCTAG
241  CGTCAGACAC  CGCGACTGCG  TGGGGCGCGC  GCTGCGGATG  AAGACATCG  ACGAGATCG
301  CCCGGAGGCG  GCGTTGGCG  AT

*D. seriata* H141a

1  CCCGGCGACGA  GGGCTTCGCG  GCTCGGGAAGC  ACGTCGCAAG  GTTCCGACTC  GGGCCAGC
61  TCCGTCAGCG  GGCGGCCGCTG  TCGAGTCGCG  GGGCTGCGCC  CGGATGGCAG  CTGGCTGAG
121  AGATTGGGGC  GCGGGCGATG  TCCAAACATCG  GGGCCGGCGC  TCCAGCGCTG  CAGCTGGCCG
181  AGGAATCGCA  GAAGAGCGAC  TAGCAGCGCG  CCCGGAGTGT  AAGGCTGGGC
241  TGAGCGTCAC  GATCCGCGAG  TGCTCGCGGG  CGGTGCTGT  GATGAACGAC  ATCGACGACA
301  TCGATCCGAG  GCGCCGGCT

*D. mutila* DAR79135

1  AGGGCTTCCG  CCGCTGGGAG  CACCTGGGCA  GGGCTGATAG  ATGTCGATCA  CGGCTTGTG
51  CTGCGTACGC  TGGCGATGACG  CGAAGGGGAG  CCGTCGCGCC  TGGCCATCT  CAGGACATC
121  GCAGGCGCTA  GAGCGGCGATC  GGCGCCGGCG  GGCGGCGCTC  ACGCCGGACG  TCGGAGAAG
181  GATGCCGGCG  GGGCGATCGC  TGGGCGGCGC  CGGGCGCTCA  CGGGCTGGTG  GAGCGACCGG
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*D. mutila* DAR79137
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**Do. vidmadera DAR78993**

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**L. theobromae DAR81024**

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**N. australe DAR79505**
Appendix D

N. australis DAR79506

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61  TTGCGGCCGG TGCGACGTGC GCGACGGCCT GCCATCCCCG TGATCGAGCA GAATCGCCCG
121  CGCCGCGTGC GTCTGGACGC GCGG

N. luteum DAR82046

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51  TGGCTTCGCG TGCGACGTGC GCGACGGCCT GCCATCCCCG TGATCGAGCA GAATCGCCCG
111  CGCCGCGTGC GTCTGGACGC GCGG

N. parvum DAR92046

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61  CGAGCGTGAC GACGCGCGCG TCACCCCGCG GTGGCGGTTT GACGTCGCGG AGCCCGTGCC
121  CATCCCGCGT GACGACCGCG TGCCCGCGCG GTGGCGGCAA CGGCGCGCGG
181  CGGCGCGCGG GCCAAGGAGA AAGCTGAGTC GAGCGCGCGG GCCGCGCGGG AGCTGAGAGG
241  CTGGCTGAGAC GTCCGCTATCC GCGGAGCTGGCGGCTGCGT GGAGCTGCGG
301  CGAGCTGGCGG CCGCGCGCGG CGGTCCGCGA TTGGCCGCGA CCGCGCGCGG
361  CCTGAGGACGA AAGCTGAGGA GCGCCGATGAA GCTCAAGGCG CCGCCGACCG TGACCTGAGA
421  CATCCCGCGT G
N. parvum E12b

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61 GTTCGACGTC GGCGAGCCCG TGCCCATCCC GCTGATCGAG CAGATCGCCC CGC6CCCGEGT
121 GGCCCCGCTG GGACCCGCCG CGGGCGCCGA GCCGGCGCAAG AAGAAGACGT AGTCAGGCCC
181 GGCCCCGCGC CCGGAGCTGA AGCCCTGGCT GAAGCTCGCC ATCCGGAGCT GCATCGCCGC
241 CGTGCCTGATG ATCGGGGACG TCGACGAGGT GGACCACCCGC GC
Appendix E – Other publications

E.1 Publication arising from other research


fungus proposed for the *Ambrosia artemisiifolia* biocontrol; spectroscopic data and absolute configuration assignment of colletoclorhin A." *Natural Product Research* **32**(13), 1537-1547.
