Grapevine Trunk Disease Management for Vineyard Longevity in Diverse Climates of Australia





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1 ABSTRACT

Grapevine trunk disease (GTD) threatens the sustainability of the Australian wine industry. Research has progressed recommendations for the management of GTD in Australian vineyards. Rapid, low-cost, DNA-based molecular diagnostic tools have been developed to detect pathogens in the vineyard and in spore traps. Pathogen spores were present all year round in association with rain, and dispersal patterns varied between climatic regions. Pruning wounds were most susceptible to infection for the first two weeks, with reduced susceptibility during late winter in the Adelaide Hills. There was no benefit in adding adjuvants (wetters or stickers) to increase coverage and improve efficacy of fungicides, and several tested biocontrols and alternative products did not provide adequate wound protection against GTD pathogens. Short-term control of Botryosphaeria dieback (BD) was achieved with remedial surgery, with grafting being the only method shown to significantly increase shoot return, and spore detection was reduced in the vineyard following remediation. There was no evidence of tolerance to GTD pathogen infection in clones and rootstocks. Microbiome studies detected relatively low abundance of young vine decline and BD pathogens along with beneficial organisms in nursery vines. Water deficit stress increased the susceptibility of young vines to BD pathogen infection, particularly under high disease pressure, but vines with a low level of infection were able to withstand disease for at least three seasons with sufficient water. These outcomes provide new information that has been extended to industry and is leading to adoption of improved strategies for managing trunk diseases, which will increase vineyard longevity in diverse climates of Australia.

2 EXECUTIVE SUMMARY

Eutypa dieback (ED) and Botryosphaeria dieback (BD) are major grapevine trunk diseases (GTDs) worldwide, causing significant yield reduction and threatening the sustainability of the $45 billion Australian wine industry. The causal pathogens infect vines through pruning wounds and colonise wood, causing dieback and, in the case of ED, stunting and yellowing of shoots and leaves, eventually killing the vine. Research led by the South Australian Research and Development Institute in collaboration with the National Wine and Grape Industry Centre (Charles Sturt University), with funding from Wine Australia and industry, has focussed on developing grapevine trunk disease management strategies for vineyard longevity in diverse climates of Australia.

Real-time loop mediated isothermal amplification (LAMP) assays were developed to detect ED and BD pathogens in infected plant materials, spore tapes and from fungal mycelia. A low cost and simple DNA extraction protocol was developed for LAMP and was found to be highly suitable for rapid DNA extraction from infected wood and other substrates. Seven real-time LAMP assays were shown to be highly specific and suitable for detecting and discriminating different ED and BD pathogens. The LAMP assays were at least twice as sensitive as conventional fungal isolation but were less sensitive than existing qPCR methods. This may offer cost effective, simple and robust diagnostic tools in the field or in limited resource environments. These assays can be used for commercial diagnostic laboratories and nurseries for the rapid detection of ED and BD pathogens from infected plant materials. Future research aims to further validate LAMP assays in the field for these and other GTD pathogens.

Spore trapping studies revealed sporadic spore dispersal that varied between regions, seasons and years. The number of samples with detection of ED pathogens was generally higher than that for BD pathogens. Rainfall was a primary factor associated with the release of either ED or BD spores, with as little as 0.2 mm of rain resulting in spore release. However, not all rain events resulted in spore release. The detection of spores in the absence of rain suggests that other climatic factors such as relative humidity or dew point may also play a role in spore release. Spores of BD pathogens were often detected throughout spring and summer, so any wounds created as part of canopy management (i.e. sucker removal, shoot trimming) may also be at risk of becoming infected by these spores. Deploying spore traps in different climatic regions will provide localised data that will assist growers in making decisions for optimal pruning time and fungicide treatments of their vineyards. DNA sequencing has identified seven species of ED pathogens, with *Eutypa lata* being prevalent in most regions. This study has led to the first report of *Diatrype stigma* and *Cryptosphaeria multicontinentalis* in Australian vineyards.

Rotorod spore traps were able to detect spores of both ED and BD pathogens, with ED predominant. ED pathogen spores were detected in and around a Shiraz vineyard with substantial dieback symptoms,

with an increasing gradient of spore numbers from the north-east corner to the southern edge of the vineyard, consistent with predominant wind from the north. Spores were detected at all heights tested from 40-250 cm, with the greatest numbers at cordon height, and were also detected in lower numbers at 40 cm, the height of the air intake orifice of the Burkard volumetric spore trap. This suggests that data from the Burkard spore may underestimate the number of spores present at cordon height. Rotorod spore traps should be run for 24 h to maximise spore detection. Spore detection was greater from control vines compared to remediated vines, but annual wound protection is still necessary to prevent infection from spores derived from inoculum sources outside of the vineyard. Rotorod spore traps provide an effective research tool for monitoring spore dispersal in vineyards under different management regimes.

Field trials in the Adelaide Hills, SA revealed that wounds were highly susceptible to *E. lata* and *D. seriata* immediately following pruning, after which the susceptibility decreased rapidly over the following 14 days. From 21 days post-pruning, susceptibility was generally negligible. This indicates that wounds are most vulnerable to infection for the first 14 days following pruning, supporting results from a previous trial in McLaren Vale, and highlighting the importance of wound protection for the first two weeks after pruning. For *E. lata*, wounds were generally most susceptible to infection when pruned early and least susceptible when pruned late. Therefore, in the Adelaide Hills, there may be an advantage in delaying pruning to later in the dormant season to minimise the risk of infection by *E. lata*. Although *D. seriata* is not prominent in the region, susceptibility of wounds to *D. seriata* did not vary greatly between pruning times, so there appears to be no real advantage in avoiding the early pruning time. Future research will focus on other regions with varying climates to provide localised recommendations for pruning and wound protection.

Cryptovalsa ampelina was recovered from cane samples in the Adelaide Hills trial with high frequency. Freshly cut canes were free of *C. ampelina*, indicating that infection occurred post-pruning, and the prevalence of *C. ampelina* fruiting bodies on dead arms in the vineyard suggested that this species may be playing a greater role in ED than previously thought. The increased prevalence of *C. ampelina* in Australian vineyards requires further investigation. It will be important to determine its prevalence and distribution in Australia, its pathogenicity and the efficacy of wound protection treatments currently recommended for GTDs.

A wound spray coverage trial indicated that there was no benefit in adding adjuvants (wetters or stickers) to increase coverage and improve efficacy of fungicides. Poor coverage and control of *E. lata* with the application of two registered fungicides at a low water rate of 200 L/ha, reiterated the importance of applying the recommended minimum water rate of 600 L/ha to achieve sufficient coverage.

A vineyard trial was established to evaluate Vinevax (*Trichoderma harzianum*) applications followed by inoculation with trunk disease pathogens from 0-28 days. The results showed that application of Vinevax to wounds led to substantial colonisation by *T. harzianum* in the canes. However, it did not provide adequate protection from infection by ED and BD pathogens, particularly in the first three days after pruning, and was less effective than the registered fungicide Gelseal (tebuconazole). Further investigation is necessary on the application of higher *T. harzianum* spore concentrations on wounds to speed up colonisation, evaluating other *Trichoderma* species and products, and screening of local isolates from Australian wine regions.

Alternative products were evaluated for protection of pruning wounds against infection by ED and BD pathogens in a series of detached cane assays. Botector (*Aureobasidium pullulans*) and chitosan both showed promise for ED, but less so for BD. Botector was subsequently evaluated in the vineyard, and there was no reduction of wound infection by either pathogen. *A. pullulans* was re-isolated at low incidence from canes, suggesting either poor or temporary colonisation. Again, further investigation is required to evaluate the efficacy of *A. pullulans* when applied at higher concentrations on wounds following pruning. Chitosan products developed for disease management should also be further evaluated, and the search for other alternative organic wound treatments should continue, to ensure the sustainable management of grapevine trunk disease.

Remedial surgery trials were established, and control of BD was reported in the short term. However, grafted vines had lower watershoot production compared to own-rooted vines, suggesting that remedial surgery may be more successful in own-rooted vines than in grafted vines. Dieback symptoms with low severity re-emerged on cordons after four years, highlighting the importance of annual wound protection to ensure the long-term success of remedial surgery for the control of BD. In a world first vineyard trial,

five methods anecdotally reported to improve watershoot growth following remedial surgery were evaluated. Only one method, grafting, significantly increased shoot production by 6 to 19%. Retaining watershoots prior to conducting remedial surgery resulted in a similar incidence of watershoots to grafting.

Visual assessment of symptoms in the vineyard indicated that the severity of dieback varied between clones. However, evaluation of a selection of clones in replicated detached cane assay experiments provided no evidence for any tolerance against *E. lata* or *D. seriata* infection between them. Therefore, it is most likely that any differences in dieback observed on vines in the field may be due to other factors such as vine age or variation in the ability of clones to tolerate other causes of dieback, such as water stress or poor soil conditions.

Assessment of established rootstock trials revealed a variation of dieback symptoms between vines planted on rootstocks and on own roots. Like the clone evaluations, rootstocks did not appear to confer tolerance on Shiraz scion canes inoculated with *E. lata* or *D. seriata* in the vineyard. Again, differences in dieback between vines on different rootstocks or own roots that were observed in the vineyard may be due to other factors such as their adaption to water stress, different soil conditions or soil-borne pathogens such as nematodes.

Two microbiome studies provided some initial insights on the microbial communities that naturally inhabit young vines from different Australian nurseries. A diverse population of the microbial communities was present in grapevine materials from different nurseries. The majority of the fungal genera identified are considered to be environmental fungi, while the remaining 8% were associated with YVD, bunch rots or fungal species with biocontrol potential that were classified as beneficial. These studies further revealed that short-term (5 min) hot water treatment used for dormant vines in Australia was ineffective in altering fungal populations but caused some variation in abundance of certain bacterial groups. Thus, investigation on the functionality of these bacterial communities is necessary to understand their role in vine health. The results generated from these studies are crucial first steps in understanding the current health status of grapevine planting materials in Australian nurseries. The new knowledge generated from this study may lead to developing new nursery practices that nurture the abundance of key beneficial microorganisms associated with young vines.

Understanding the threshold of infection and the stress conditions that have the potential to trigger disease expression in vineyards will contribute to the development of management strategies to mitigate GTD infections in nurseries and young vineyards. This study initially focused on the evaluation of a published inoculation method for standardising different levels of BD infection. The method was found to be suitable for inoculating different inoculum levels in vines, without significantly impacting plant viability. The qPCR assays further allowed the accurate quantification of BD infection from inoculated vines. These methods enabled the production of the large number of grapevine canes needed for pot experiments and could be adopted for potential future infection threshold experiments in the vineyard. This study provided key evidence that water deficit can increase the susceptibility of young vines to *N. luteum* infection, particularly when the vines are under high disease pressure. Furthermore, vines with a low level of infection were able to withstand the disease for at least three seasons when given sufficient water.

Extension of project outcomes has been delivered to industry through 10 industry journals and articles, 36 conference papers and 53 workshop presentations and media interviews. In addition, project team members have contributed to 19 scientific publications, a book chapter and a Best Practice Management Guide. We anticipate a further four scientific publications based on results from the current project. Australia remains a world leader of research on management of grapevine trunk diseases.

3 BACKGROUND

Eutypa dieback (ED) and Botryosphaeria dieback (BD) are major trunk diseases worldwide, causing significant yield reduction and threatening the sustainability of Australian vineyards. Fungal species of the Diatrypaceae and Botryosphaeriaceae infect vines primarily through pruning wounds and colonise wood, causing dieback and death. Trunk diseases ranked in the top five priority diseases of the Australian winegrape industry (GWR 08/04) and are becoming more prevalent as vineyards mature (Sosnowski and Wicks 2012), threatening the sustainability of the wine industry, which contributes $45.5 billion per annum to the Australian economy (Gillespie and Clarke 2019). Yield losses of up to 1,500 kg/ha have been reported in Australia for Shiraz vineyards (Wicks & Davies 1999) and, in California, economic losses of at least US$260 million per annum have been attributed to trunk diseases (Siebert 2001).

In the case of ED, spores of *Eutypa lata* and related diatrypaceaous species (Trouillas *et al*. 2011) are released from fruiting bodies on dead, infected wood and are spread by rain-splash and wind. Infection occurs when spores land on exposed pruning wounds, germinate and eventually colonise the cordons and trunk, causing cankers and a characteristic wedge of dead tissue (Figure 3.1 a&b) and cordon dieback (Figure 3.1 c). The fungus produces toxic metabolites which are translocated to the foliage, resulting in stunted shoots, distortion and necrosis of leaves, reduced bunch size and uneven ripening (Figure 3.1 d-f; Moller & Kasimatis 1981, Tey-Rulh *et al*. 1991, Molyneux *et al*. 2002). If not controlled, the fungus eventually kills infected vines.

In the case of BD, spores of botryosphaeriaceous species are released from fruiting bodies embedded in diseased woody parts of vines as well as in wood debris left in the vineyard after pruning (Figure 3.2a). Similarly to ED, spores are dispersed by rain from the inoculum produced within the vineyard. Infection of grapevines occurs primarily through wounds made during pruning or via other mechanical damage. Wood necrosis is often associated with pruning wounds and when the wound is cut open, wood staining of various shapes is visible that often reflects the position of pathogen entry into wounds. The common internal wood symptom observed is a wedge-shape necrosis that is similar to ED, however the vines do not show the foliar symptoms characteristic of that disease (Figure 3.2b-d) (Úrbez-Torres 2011). In warmer and more humid climates such as those experienced in the Hunter Valley, Botryosphaeriaceae species have also been associated with bunch rots (Figure 3.2e) (Wunderlich *et al*. 2011).

There is conflicting information from abroad on dispersal of *E. lata* and Botryosphaeriaceae spores in vineyards (Gramaje *et al*. 2018). Burkard spore traps were deployed in four Australian wine regions from 2013 to 2015 (SAR 1205; Billones-Baaijens *et al*. 2017, Sosnowski *et al.* 2017). Rapid and highly sensitive DNA-based molecular tools were developed to detect and quantify ED and BD pathogens on spore tapes. Spores were detected throughout the year, but the seasonal dispersal and number of spores differed between pathogens and regions. Rainfall was confirmed as the primary factor that triggers the release of spores. The prevalence of rain varies seasonally between different climatic regions, and therefore will influence the timing of spore release. Wine regions in Australia cover a wide area with highly diverse climates, so it is important to continue monitoring spore dispersal patterns so that localised recommendations can be provided. Further development of in-field protocols for detecting GTD spores is required to align with real-time spore trap technology being developed abroad. In the future, real-time spore monitoring could provide a decision-making tool for growers, leading to improved management of GTD.

Wound susceptibility to trunk pathogens varies during the pruning season and between environments and can persist for up to 12 weeks according to overseas research on apricots and grapevines (Gramaje *et al.* 2018). Vineyard trials in Australia have provided new information on the timing and duration of wound susceptibility to ED and BD pathogens (SAR 1205; Ayres *et al*. 2016, Sosnowski *et al.* 2017). Results revealed that wounds were highly susceptible for two weeks following pruning, after which the susceptibility often decreased sharply, at varying rates for each pathogen evaluated and between years. In general, wounds were more susceptible to *E. lata* (ED) when pruned early (June) than at the later pruning times in July and August. Susceptibility to *N. luteum* (BD) was lowest at the late pruning time, and there was no difference in susceptibility to *D. seriata* (BD) between pruning times. Detached cane assays in the greenhouse showed that wound susceptibility did not differ between cultivars commonly grown in Australia. These results highlighted the importance of protecting pruning wounds for at least two weeks post-pruning.



Figure 3.1. Symptoms of eutypa dieback in grapevines: (a) external canker extending from wound on cordon, (b) wedge of dead wood in trunk cross-section, (c) cordon dieback, (d) mature vine with stunted shoots (e) single stunted shoot with cupped leaves with necrotic margins and (f) uneven ripening and reduced bunch size.

It is now important to evaluate the susceptibility of wounds to ED and BD pathogens in other regions to provide localised recommendations for the diverse range of climates in Australia.

Pruning wound fungicides are registered for control of GTDs and some can be applied with sprayers (SAR 1001; Ayres *et al.* 2017b; Sosnowski and Mundy 2019). Curative and preventative properties of fungicides are evident for both ED and BD pathogens (SAR 1205; Ayres *et al*. 2017a, 2022). However, the ability of adjuvants to maximise fungicide coverage on wounds is unknown. Only one organic alternative is registered for wound protection, but evaluation of the critical timing for biocontrol application and efficacy of natural alternative compounds is necessary to provide more options for organic and sustainable disease management.



Figure 3.2. Symptoms of Botryosphaeria dieback in grapevines. Wood symptoms including (a) pycnidia on the wood surface, (b) wedge of internal staining, (c) internal canker extending from the cordon down into the trunk, (d) characteristic short, stunted shoots and missed spur positions, and (e) bunch rot symptoms.

Remedial surgery, where infected wood is removed and vines reworked from lower shoots in healthy wood, is the only effective method for managing ED-affected vines (SAR 06/01; Sosnowski *et al.* 2011). Remedial surgery was evaluated as a curative control strategy for grapevines with BD (SAR 1205; Savocchia *et al.* 2017, Sosnowski *et al.* 2017). Vines recovered and were able to produce new shoots after remedial surgery, although grafted vines tended to produce shoots from the rootstock rather than the scion. The severity of dieback in untreated vines increased by 5-10% each year, but no symptoms were recorded on remediated vines. Continued monitoring of these vines would determine longer-term success of the strategy. There is also a need to investigate remedial surgery for grafted vines and evaluate novel methods of water shoot induction to try to improve the success of the technique.

There is evidence of cultivar tolerance to trunk disease (SAR 1205; Sosnowski *et al.* 2016, Sosnowski *et al.* 2017, 2022). Visual assessment for symptoms of trunk disease in a mature germplasm cultivar collection revealed that cultivars ranged markedly for symptom severity. Further evaluation of disease progression by inoculation of pruning wounds in the vineyard, and on detached canes, confirmed that some cultivars appeared to be more tolerant to infection by ED and BD pathogens. There was some correlation between cane width, lignin content and colonisation, which was consistent with findings from previous research (Rolshausen *et al.* 2008, Pouzelet *et al.* 2014, Hamblin 2015). A preliminary visual assessment of symptoms in a clonal germplasm collection indicated that the severity of trunk disease varied considerably amongst the clones of a number of cultivars (Sosnowski *et al.* 2017). This warrants further investigation to determine whether certain clones might be recommended for future plantings, offering an additional means of disease management.

Worldwide reports on the role of nursery propagation material in disease spread have raised concerns with regard to their health status in Australia (Gramaje and Armengol 2011, Gramaje *et al.* 2018). With the development of new, highly sensitive molecular diagnostic tools, and concerns about the potential

spread of disease in nurseries, it is important to establish the infection thresholds in propagation material and growing conditions that may lead to subsequent disease symptoms and vine decline.

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4 PROJECT AIMS

Surveillance of inoculum dispersal in selected climatic regions
Determine the presence and quantity of spores of GTD pathogens in relation to weather throughout the year in selected climatic regions of Australia and develop an inexpensive field-based diagnostic tool for spore tape analysis.

Determine pruning wound susceptibility under different climatic conditions
Determine the length of time for which grapevine wounds are susceptible to infection by spores of GTD pathogens at key times during the pruning season in different climatic conditions.

Maximise wound coverage by fungicide and evaluate the efficacy of organic alternatives for pruning wound protection
Evaluate the addition of adjuvants to fungicide to improve spray coverage on wounds and the efficacy of fungicide, and evaluate the efficacy of biocontrol and natural alternatives for sustainable control of GTDs.

Optimise remedial surgery techniques to control GTDs
Monitor established and new remedial surgery trials to manage BD-affected vines and evaluate novel methods of water shoot induction to improve the success of the technique.

Assess susceptibility of clones and rootstocks to GTD
Assess clones of common cultivars and scion/rootstock combinations for symptoms of GTD.

Investigate infection thresholds of BD in propagation material
Quantify the amount of GTD pathogens in nursery propagation material followed by planting under various stress conditions to monitor for disease expression.

5 LAMP ASSAYS FOR RAPID DETECTION

5.1 Introduction

PCR-based techniques are very powerful tools for pathogen detection and disease diagnosis due to their accuracy and sensitivity compared to conventional plant pathology techniques. Several PCR-based techniques have been developed to detect and quantify grapevine trunk disease (GTD) pathogens in different samples. Ridgway *et al*. (2011) and Spagnolo *et al*. (2011) developed multi-species primers for detecting Botryosphaeriaceae pathogens from environmental samples in New Zealand and Italy, respectively. Quantitative PCR (qPCR) assays were also developed for the detection and quantification of Petri disease pathogens, *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (Martin *et al*. 2012, Pouzoulet *et al.* 2013) and *Eutypa lata* and *Diplodia seriata*-complex (Pouzoulet *et al.* 2017).

In Australia, highly specific and sensitive qPCR assays were developed to detect and quantify multiple Diatrypaceae and Botryosphaeriaceae species from environmental samples (SAR 1205; Sosnowski *et al.* 2017; Billones-Baaijens *et al*. 2018). These molecular tools fast-tracked the analysis of spore trap samples and allowed quantification of Eutypa dieback (ED) and Botryosphaeria dieback (BD) spores from vineyards as part of comprehensive spore trapping studies in SAR 1205 (Sosnowski *et al*. 2017). These molecular tools were further found to be suitable for detecting GTD pathogens from infected plant material as shown in the infection threshold experiments (Section 11.2). While PCR-based techniques allow the detection and quantification of very low amounts of DNA in a wide range of samples, they are labour intensive and require expensive equipment and highly skilled staff to perform the analyses. These assays are also highly sensitive to biological contamination and need to be performed under sterile conditions, and thus, are not suitable for field-based applications. Development of rapid, simple but sensitive and accurate molecular assays for field use could allow grape growers to conduct their own real-time monitoring of GTD spores in the vineyard. The real-time detection of GTD spores can assist in making decisions for the timing of pruning and fungicide applications to grapevines. Field-based molecular tools would allow rapid diagnostics for infected vines in the field without the need for samples to be sent to diagnostic laboratories. These tools would also be useful for grapevine nurseries in detecting GTD pathogens as part of their quality control system.

Loop mediated isothermal amplification (LAMP) developed by Notomi *et al*. (2000) is a rapid, sensitive but simple method for the detection of a wide variety of pathogens from humans, animals and plants. This method is conducted under constant temperatures ranging from 60 to 65°C, thus, LAMP assays can be performed using simple heating equipment such as a heat block or water bath (Notomi *et al*. 2000). LAMP is also highly specific, since this technique relies on four to six primers that target six to eight regions within the target DNA sequence (Notomi *et al*. 2000; OptiGene 2017). LAMP assays using four primers contain two outer primers (F3 and B3) and two inner primers (FIP and BIP) while assays using six primers have the addition of two loop primers (LoopF and LoopB) to speed up amplification (OptiGene 2017). Purified DNA is also not essential for LAMP, as it is known to be less sensitive to PCR inhibitors (Tomlinson *et al*. 2007), making it suitable for field-based testing.

LAMP products can be detected in different ways including visualisation by gel electrophoresis, (Tomlinson *et al.* 2010), colour change in the reaction, such as colorimetric LAMP (Liu *et al*. 2014) or real-time fluorescence detection using the Genie® II instrument (OptiGene, U.K.; Tomlinson *et al*. 2010). Given that these methods are rapid and simple but are also accurate compared to other molecular tools, LAMP is now widely used for rapid detection of different grapevine pathogens including *Botrytis cinerea* (Tomlinson *et al*. 2010), the powdery mildew pathogen, *Erysiphe necator* (Thiessen *et al*. 2016), grapevine yellows phytoplasmas (Kogovsek *et al*. 2017) and the downy mildew pathogen, *Plasmopara viticola* (Marimuthu *et al*. 2019). The detection of GTD pathogens *P. chlamydospora* and *E. lata* (Woodhall *et al*. 2019) and *D. seriata* and *N. parvum* (Cortez *et al*. 2019) has also been reported recently but protocols for the LAMP assays are unpublished to date.

The objective of this study was to develop LAMP assays for the rapid detection of ED and BD pathogens in different samples. Various LAMP methods were evaluated for their suitability as (a) field-based molecular tools for monitoring GTD airborne spores in vineyards; and (b) rapid detection for ED and BD pathogens in different grapevine plant materials. These methods were compared with the existing laboratory methods (fungal isolations on artificial media and qPCR) for the detection of the most prevalent Botryosphaeriaceae and Diatrypaceae pathogens in Australian vineyards.

5.2 Methods

5.2.1 LAMP primer development and pre-selection

Published reference sequences (three each) of the ribosomal RNA (rRNA), β-tubulin and translation elongation factor 1-alpha (tef1α) genes for *E. lata*, *E. leptoplaca*, *Crytovalsa ampelina*, *Eutypella citricola*, *Eu. microtheca, Diplodia mutila*, *D. seriata*, *Neofusicoccum australe*, *N. luteum*, and *N. parvum* were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/) and used for primer development.

One representative sequence (400-500 bp) for each species was uploaded in PrimerExplorer Software Version 5.0 (Eiken Chemical Co., Ltd) to generate the outer primers (F3 and B3), inner primers (FIP and BIP) and loop primers (Loop F and Loop B) for each target species. The specificity of each individual primer was aligned to the representative sequence for each species by ClustalW, using Mega 6 (Tamura *et al.*, 2007) to manually check their theoretical specificity to their target species. Those primers lacking specificity were discarded while the highly heterogeneous primers were selected for *in vitro* testing.

5.2.1.1 DNA samples

Genomic and crude fungal DNA samples of the Diatrypaceae (12 isolates) and Botryosphaeriaceae (13 isolates) from the DNA sample collection at the NWGIC, Charles Sturt University (CSU) were used for optimisation and specificity testing of the primers (Table 2). These DNA samples were previously identified by partial DNA sequencing of the rRNA and β-tubulin genes (Pitt, *et al.* 2010; Trouillas *et. al.* 2011; Wunderlich, *et al.* 2011).

All plant genomic DNA used for all LAMP assays was extracted using the Qiagen Plant Pro Kit (Qiagen, USA) following the manufacturer's protocol. Wood samples were cut into ~1 cm sections before surface-sterilising with 70% ethanol for 2 min, rinsed twice with sterile deionised water (SDW) for 1 min and air-dried inside the laminar flow for ~30 minutes. All samples were homogenised using a sterile coffee grinder before transferring sub-samples into 5 mL tubes and stored at -80°C for 7-14 days until freeze-drying. All wood samples were freeze-dried (Christ, John Morris Scientific, U.S.A.) for 36 h and dried samples were pulverised at 20 Hz for 2 min 30 s using 10 mL grinding jars attached to a TissueLyser II (Qiagen, U.S.A.). The ground wood (100 mg) was transferred into a sterile 2 mL tube for the DNA extraction using the DNEasy Plant Pro Kit following the manufacturer's protocol (Qiagen, USA).

5.2.1.2 Design of gBlocks gene fragments

To generate the standards for LAMP assays, the three to five reference sequences for each target species were further aligned by ClustalW using MEGA7 (Kumar *et al*. 2016) to design a chemically-synthesised single copy of the β-tubulin gene for all Diatrypaceae species and *Neofusicoccum parvum*, while *D. seriata* was based on translation elongation factor 1-alpha (tef1α). The 410-500 bp gBlocks® Gene Fragments (Integrated DNA Technologies, USA) were developed for each target species. The annealing sites for all primers are within the 75 and 385 bp of each fragment. A 10 ng/µL solution was prepared by resuspending the 500 ng lyophilised gBlocks® in 50 µL of TE Buffer (Tris and EDTA, pH 8.0; Sigma Aldrich) following the manufacturer's recommendation.

5.2.1.3 Primer selection

All LAMP primers were initially screened using a thermal cycler (C100 Thermal Cycler, BIO-RAD Laboratories, Pty, Ltd., U.S.A.). The 25 µL reactions contained 1× Isothermal mastermix (ISO-DR001, Optigene Ltd., UK), 0.2 µm each of F3 and B3 primers, 0.4 µm each of Loop F and Loop B primers and 1.6 µm each of FIP and BIP primers and 5-10 ng of gDNA for each species. A non-template control (H_2O) was included for each assay. The isothermal amplification was performed in a gradient thermal block at a temperature range of 61°C to 68°C for 30 min. Following amplification, 5 µL of each product was separated by electrophoresis (10 V/cm for 50 min) in a 1.5% agarose gel (Bioline, U.K.) in 1× TAE buffer. Gels were stained with 1× GelRed nucleic acid gel stain (Biotium, U.S.A.) and visualised under UV light using a Gel Doc XR+ Imaging System (BIO-RAD Laboratories, Pty, Ltd., U.S.A.). The positive reaction was visualised as a ladder-like pattern on a 1.5% agarose gel. The optimum temperature for each assay was determined based on the clarity and intensity of ladder-like patterns and was used for real-time LAMP assays using the Genie II instrument.

5.2.1.4 Real-time LAMP assays using Genie II instrument

LAMP primers that produced clear ladder-like patterns in 1.5% agarose gels were further tested by real-time LAMP using the Genie II instrument (Optigene Ltd, UK). Each LAMP assay was performed in 0.2 mL Genie tubes (OptiGene, Ltd, UK). The 25 µL reactions contained 1× Isothermal mastermix (ISO-DR001, Optigene Ltd., UK), 0.2 µm each of F3 and B3 primers, 0.4 µm each of Loop F and Loop B primers and 1.6 µm each of FIP and BIP primers and 5-10 ng of template gDNA for each species. The isothermal amplification for each primer set was performed at 63°C, 65°C and 67°C for 30, 45 and 60 min. For each assay, two controls were included: a) non-template control (NTC, H_2O) and b) positive control (gBlock, 200 pg). The optimum temperatures were determined based on the average time taken to generate a positive result (Tp values; Tomlinson *et al*. 2010). All assays were repeated three times.

5.2.1.5 Assays on gBlocks

All primer sets were tested for their sensitivity using the serial dilutions of their corresponding gBlocks as templates. The gBlocks for each species were serially diluted from 100 pg to 10 fg and 2 µL of each dilution was used as a template for real-time LAMP using the Genie II instrument. In all assays, sterile and DNA-free water was used as the negative control. All assays were performed using three replicates and repeated once following the optimum conditions for each assay.

5.2.1.6 Assays on fungal genomic DNA

All primer sets were further tested using serially-diluted gDNA. For each species, 2 ng to 2 pg of gDNA per reaction was used as a template for real-time LAMP. In addition to the 10-fold dilutions, lower concentrations of gDNA (1 pg and 500 fg) were also included. All assays were performed using three replicates and repeated once following the conditions previously described gBlocks.

5.2.1.7 Assays on mixed DNA (plant and fungi)

To test the effect of plant DNA on the sensitivity of the assays, LAMP assays for each primer set were performed using gDNA extracted from a *V. vinifera* vine (cv. Shiraz) that tested negative to BD and ED by qPCR assays (Section 11.2). The templates for each LAMP assay contained plant DNA (~60 ng) that was spiked with 2 ng, 200 pg, 20 pg and 2 pg of each target fungal gDNA. In all assays, fungal DNA alone (2 ng) and sterile water were used as positive and negative controls, respectively. Each assay was performed using three replicates and repeated once. The assays were further repeated using crude plant extracts instead of purified DNA.

5.2.2 Development of rapid and field-based DNA extraction protocol

Three commercial rapid DNA extraction kits: (1) Plant Material DNA extraction kit, Optigene, UK; (2) QuickExtract™ DNA extraction solution, Lucigen; USA; (3) Prepman Ultra, Applied Biosystems, UK, were initially tested for their suitability for rapid DNA extractions from infected wood. One published protocol (Chomczynski and Rymaszewski, 2006) was further included in the initial tests. Preliminary tests were conducted for each protocol using frozen wood shavings (100 mg) from a grapevine wood sample that tested positive to *Eu. microtheca* by fungal isolations. To determine the efficiency of each protocol, 5 µL of each crude plant extract was used as a template for real-time LAMP assays using the Mt2 primers specific for *Eu. microtheca*, following the methods described previously for gBlocks and gDNA.

5.2.2.1 PEG Rapid DNA extractions from wood

A rapid plant DNA extraction method, now referred to as PEG rapid DNA extraction, was further developed by combining the protocols from the Optigene Plant Material extraction kit and Chomczynski and Rymaszewski, (2006) with numerous adaptations. This protocol gave consistent results during the initial tests. This protocol, which is described in detail below (Figure 5.1), was further tested using a one-year-old grapevine cane vacuum-inoculated with 30,000 *N. luteum* spores (Section 11.2.2) and *E. lata* (AD15) and *C. ampelina* (SH2O) mycelial plugs. The bark was removed from each vine before surface- sterilising using 70% EtOH for 2 min, and rinsed twice with sterile water for 2 min. For the vacuum-inoculated vine, 200-500 mg of tissue was grated from the basal end of the vine using a sterile cheese grater. The grated tissue was placed in a 2 mL tube and stored at -20°C for 5-7 days and used for rapid DNA extractions. For *E. lata* and *C. ampelina* inoculated vines, ~1 cm wood that spanned the area of the inoculation point was excised and 200-500 mg of tissue was grated from the margin between

the green and necrotic tissue. All grated tissue was stored as above and used for rapid DNA extractions. For extraction, ~100 mg of grated wood was transferred into a sterile 2 mL screw cap tube containing one ceramic ball (1/4" MP Biomedical Australasia Pty Ltd) and ~10 pcs of stainless-steel ball bearings (1 mm). Alkaline polyethylene glycol (PEG) lysis buffer pH 13.5 (Chomczynski and Rymaszewski, 2006) was added and the tube was vortexed twice for 1 min and allowed to stand for 1-3 min at room temperature. The lysate was diluted five-fold using sterile DNA-free water, and 5 µL of the diluted sample was used as a template for real-time LAMP. Each assay was performed using three replicates and repeated once using gBlocks (200 pg) and sterile water as positive and negative controls, respectively.

5.2.2.2 PEG Rapid DNA extractions from spore tapes

The PEG rapid DNA extraction with some adaptations was further tested for its suitability for rapid DNA extractions from spore tapes. Melinex tapes (2 × 3 cm) were inoculated with different concentrations of *D. seriata* conidia (14,000, 7,000, 1,400 and 750) and *E. lata* ascospores (40,000, 20,000, 10,000 and 5,000) following the protocol described by Billones-Baaijens *et al.* (2018). Inoculated tapes were placed in 2 mL tubes and stored at -20°C for DNA extractions. Crude DNA was extracted from each spore tape using the methods described for PEG rapid DNA extraction but using Ballotoni beads instead of stainless ball bearings. The lysate was diluted as described for rapid plant DNA extractions and 5 µL of the diluted sample was used for real-time LAMP for *Diplodia* spp. and *E. lata*. Each assay was performed using three replicates and repeated once using gBlocks (200 pg) and sterile water as positive and negative controls, respectively.

Due to poor amplification, the rapid DNA extractions from tapes inoculated with *E. lata* ascospores were further modified to improve efficiency. After vortexing, the lysates were incubated at either 70°C or 98°C for 10 minutes with or without Proteinase K (10 µl, Promega, USA). The lysate was diluted as described in plant DNA extractions and 5 µL of the diluted sample was used for real-time LAMP for *E. lata*. Each assay was performed using three replicates and repeated once using gBlocks (200 pg) and sterile water as positive and negative controls, respectively.

5.2.2.3 PEG Rapid DNA extractions from fungal mycelia

The PEG rapid DNA extraction was further tested for its suitability for rapid DNA extractions from fungal mycelia. Four and ten isolates with *E. lata* and *C. ampelina* morphology, respectively, were grown on PDA for seven days at 25°C and used for DNA extractions. Crude fungal DNA was extracted from the mycelia using the PEG rapid DNA extractions. 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 PEG lysis buffer (pH 13.5). Mycelial suspensions were vortexed for 30 seconds and the lysate was diluted as described above in wood DNA extraction. A real-time LAMP assay was performed for each DNA sample (2 µL) using the *E. lata* and *C. ampelina* species-specific primers following the methods described previously. Each assay was performed using three replicates and repeated once using gBlocks (200 pg) and sterile water as positive and negative controls, respectively.



Figure 5.1. Development of a rapid and low-cost DNA extraction protocol for wood samples suitable for LAMP assays. Inoculated cane was peeled and surface sterilised (1) and grated using a sterile grater (2); the grated tissue (3) was placed in a lysis tube containing ceramic and stainless steel balls (4) before adding 1 mL of PEG lysis buffer (5) the tube was shaken for two minutes and the lysate was diluted five-fold using sterile water (6); the diluted sample was transferred into the Genie strip tube (7) and analysed by real-time LAMP using the Genie II instrument (8). The positive amplification is based on the increase in the amplification curve displayed on the Genie II screen (9).

5.2.2.4 Performance evaluation of real-time LAMP

The performance of real-time LAMP assays using genomic DNA (G-LAMP) and crude DNA (C-LAMP) was evaluated by comparing the results of each LAMP assay to quantitative PCR (qPCR) and fungal isolations.

5.2.2.5 G-LAMP vs. quantitative PCR (qPCR)

The performance of G-LAMP assay was compared to qPCR using the BD genus-specific primers for *Diplodia* and *Neofusicoccum* spp. The gDNA samples extracted from dormant Shiraz canes previously vacuum-inoculated with 300, 3,000 and 30,000 spores of either *N. luteum* or *D. seriata* as described in infection threshold experiments (Section 11.2.2) were used for the evaluation. All DNA samples were analysed by qPCR prior to LAMP assays to determine the number of pathogen DNA copies per reaction. The genomic DNA samples from wood (5 µL) were used as templates for testing the primers Dpef1 and Nbt1 by real-time LAMP using Genie II with three technical replicates per sample. Positive detection for each primer was compared with the results obtained from qPCR.

5.2.2.6 G-LAMP vs. fungal isolations

The performance of LAMP assay using gDNA was compared to fungal isolations. One-year-old canes artificially inoculated with *E. lata* ascospores (n=9) and non-inoculated controls (n=8) during winter 2019 for the pruning wound susceptibility trial (Section 7.2) were used for the analysis. The bark was removed from each cane, surface-sterilised using 70% EtOH for 2 min, and rinsed twice with SDW for 2 min. The top 2 cm of the surface-sterilised cane starting immediately below the inoculation point was cut and homogenised into smaller fragments using a sterile coffee grinder and 10 pieces of the wood chips were randomly selected and plated onto two PDA-C plates at five wood chips per plate. Plates were incubated at 25°C total darkness for 7-14 days and observed for ED pathogen growth. Approximately 1 g of the remaining wood chips was placed into 2 mL sterile tubes and stored at -80°C until processing for DNA extractions. All frozen samples were freeze-dried (Christ, John Morris Scientific, U.S.A.) for ~36 h followed by homogenisation using 10 mL grinding jars attached to a TissueLyser II (Qiagen, U.S.A.) at 20 Hz for 2 min 30 s. The pulverised wood (100 mg) was transferred into a sterile 2 mL tube and used for DNA extractions using the DNeasy Plant Pro Kit (Qiagen, USA) following the manufacturer's protocol. Each gDNA sample from wood (5 µL) was used for testing the five ED species-specific primers by real-time LAMP using the Genie II using three technical replicates per sample following the methods described previously. Positive detection for each primer was compared with the results obtained from fungal isolations.

5.2.2.7 C-LAMP vs. fungal isolations

The performance of LAMP assay using crude DNA was further compared to fungal isolations. One-year-old canes (n=110) artificially-inoculated with *E. lata* ascospores during winter 2020 for the Botector organic alternative products trial (Section 8.3.1.2) were used for the analysis. All canes were processed and surface-sterilised as described above (Section 5.2.2.1). Using flame-sterilised secateurs, most of the dead top was cut from the cane, leaving a few millimetres of dead wood above the interface with live tissue. The canes were then split in half longitudinally. One longitudinal section was further cut into segments taken from the margin between the live and dead tissue. Segments were plated onto two PDA plates with five wood segments per plate. Plates were incubated at 25°C under fluorescent light for 12 h each day seven days and assessed for the presence of *E. lata*. A sample was considered positive to *E. lata* re-isolation if at least one segment resulted in the growth of *E. lata* on PDA. The remaining half of the sample was processed for LAMP by grating the section from the margin between the live and the dead tissue using a sterile cheese grater. The grated tissue was transferred into a 2 mL screw cap tube (Corning 430659) and stored at -20°C. All samples were shipped to Wagga Wagga, NSW in dry ice. Approximately 100 mg of frozen tissue was used for PEG rapid plant DNA extractions as described in (Section 5.2.2.1). The real-time LAMP assay for *E. lata* was performed using 5 µL of the crude plant extracts as a template. All samples were analysed in duplicates with *E. lata* gBlocks (100 pg) and sterile water as positive and negative controls, respectively. A sample was considered positive to LAMP if at least one out of two LAMP reactions was amplified.

5.2.2.8 Primer test using colorimetric LAMP

The genus-specific primers for BD pathogens (Def and Nbt2) and *E. lata* species-specific primer (Elt1) were further tested for their suitability using colorimetric LAMP. The LAMP was performed in 0.2 mL clear PCR 8 Snapstrip tubes (Interpath Services). The 25 µL reactions contained 1× WarmStart Colorimetric LAMP 2× Master Mix (New England Biolabs, UK), 0.2 µm each of F3 and B3 primers, 1.6 µm each of FIP and BIP primers and 10-20 ng of gDNA of *D. seriata (B135)* and *N. parvum* primers. For *E. lata* primers Elt1, 5 µL of gBlocks serially-diluted from 1 pg to 10 fg were used for the test. The isothermal amplification for each primer was performed in a thermal cycler (C100 Thermal Cycler, BIO-RAD Laboratories, Pty, Ltd., U.S.A.) using the following conditions: amplification at 65°C for 30, 45 and 60 minutes at two tubes per sample. For each assay, two controls were included: a) non-template control (NTC, H_2O); b) non-target DNA negative control. Following amplification, the tubes were removed from the thermal cycler and examined visually for a colour change. A yellow colour indicated a positive reaction while a negative reaction was pink.

Colorimetric LAMP assays for genus-specific primers for BD pathogens (Dpef and Nbt1) and *E. lata* species-specific primer (Elt1) were further repeated with the addition of loop primers for each assay. All colorimetric LAMP assays were performed as described above with the addition of 0.4 µm each of Loop F and Loop B primers for their respective target species. Each assay was performed using three replicates and repeated once using sterile water as negative controls.

Each colorimetric LAMP assay was further tested using gDNA and crude DNA extracted from infected vines using the extraction methods previously described for real-time LAMP, and 5 µL for each type of DNA was used as templates. Each assay was performed using three replicates and repeated once using gBlocks (200 pg) as positive controls while sterile water and a 1:5 dilution of PEG buffer were used as negative controls.

5.3 Results

5.3.1 Primer development

Ten primer combinations each for the BD pathogens belonging to the genus *Diplodia* and *Neofusicoccum* spp. were initially identified based on the β-tubulin gene and translation elongation factor 1-alpha (tef1α) sequences, respectively. Of the 10 primers identified, only three to four primers were heterogeneous and specific to the genus *Diplodia* and *Neofusicoccum* and were selected for the subsequent tests. No species-specific primers for Botryosphaeriaceae spp. were identified from any of the other primers generated.

Five primer combinations each for the five ED pathogens *E. lata, C. ampelina*, *E. leptoplaca, Eu. citricola*, and *Eu. microtheca* were initially identified based on the β-tubulin gene sequences. Theoretical specificity tests showed two to three primers for each species were heterogeneous and specific and were selected for subsequent tests. No genus-specific primers for the Diatrypaceae spp. were identified from any of the other primers generated.

5.3.2 Primer selection

5.3.2.1 Botryosphaeriaceae genus-specific primers

Typical ladder-like patterns were observed in two out of four genus-specific primers for *Diplodia* spp. (Dpef1 and Dpef2) and one out of three for *Neofusicoccum* spp. (Nbt2) when visualised using 1.5% agarose gel and were selected for subsequent tests. The ladder-like patterns were not observed from any of the non-template controls (NTC; Figure 5.2).

5.3.2.2 Diatrypaceae species-specific primers

Typical ladder-like patterns were further observed for the ED species-specific primers for *E. lata* (Elt1, Elt2 and Elt3), *C. ampelina* (Cmp1 and Cmp2) and *E. leptoplaca* (Lp2), *Eu. citricola* (Ct2) and *Eu. microtheca* (Mt2) when visualised using 1.5% agarose gel. The ladder-like patterns were also not observed from any of the NTCs.

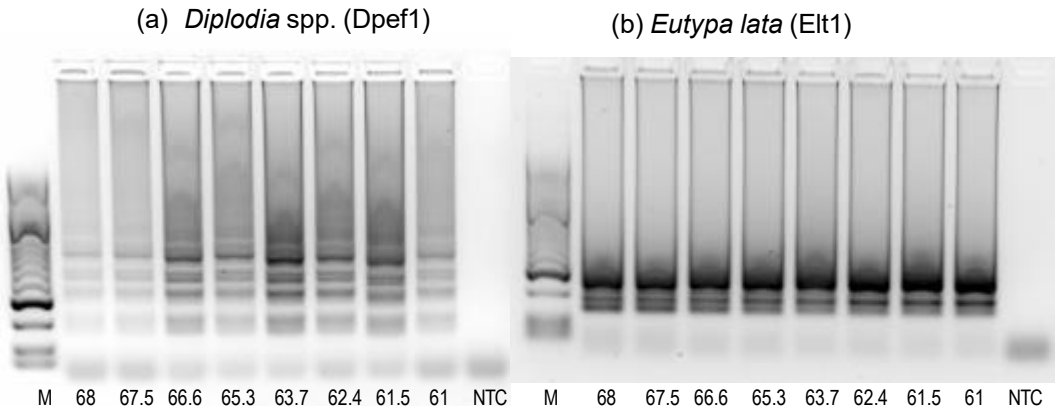


Figure 5.2. LAMP assays using *Diplodia* genus-specific (Dpef1) and *Eutypa lata* species-specific (Elt1) primers. Positive reactions are visualised as ladder-like patterns in 1.5% agarose gel electrophoresis. Isothermal temperatures (°C) are indicated in each lane; M – indicates 50 bp DNA marker; NTC indicates non-template controls.

5.3.3 Real-time LAMP assays using Genie II instrument

The two genus-specific primers for *Diplodia* (Dpef1 and Dpef2) were able to amplify *D. mutila* and *D. seriata* gDNA and qBlocks by real-time LAMP using Genie II. An increase in fluorescence was observed during amplification for both primers while no fluorescence was detected for any of the non-template controls (Figure 5.3a). The primer set Dpef1 showed faster amplification than Dpef2 (data not shown) and was selected for the subsequent tests. The genus-specific primer for *Neofusicoccum* spp. (Nbt2) further amplified *N. australe*, *N. luteum* and *N. parvum* gDNA and qBlocks using Genie II. The optimum isothermal temperature was 67°C for the two primer sets, respectively (Table 5.1).

The ED species-specific primers further amplified gDNA and qBlocks by real-time LAMP using Genie II with increased fluorescence observed for all primer sets during amplification of their respective target DNA while no fluorescence was detected in any of the NTCs. The *E. lata* primer Elt1 primer (Figure 5.3b) and *C. ampelina* primer Cmp1 with lower Tp values were selected for the subsequent tests. The optimum isothermal temperatures for each primer are shown in Table 5.1.

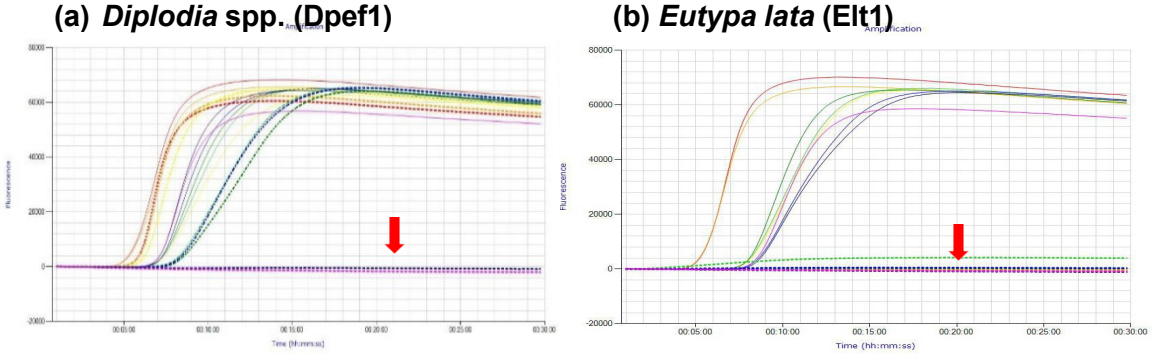


Figure 5.3. Representative amplification curves for real-time LAMP assays using Genie II. (a) *Diplodia* genus-specific primers Def1; and (b) and *Eutypa lata* species-specific primers Elt1. Each graph showed a steep increase in fluorescence during amplification of their respective target DNA while no fluorescence was detected for the non-template control (arrows).

Table 5.1. LAMP primers for Botryosphaeriaceae and Diatrypaceae species, their target genes and optimum isothermal temperatures.

Target species	Primers	Target gene	Optimum temperature
Botryosphaeriaceae			
Diplodia spp.	Dpef1	Elongation factor (tef)	67°C
Neofusicoccum spp.	Nbt1	β-tubulin	67°C
Diatrypaceae			
Cryptovalsa ampelina	Cmp1	β-tubulin	65°C
Eutypa lata	Elt1	β-tubulin	65°C
E. leptoplaca	Elp2	β-tubulin	67°C
Eutypella citricola	Ct2	β-tubulin	67°C
Eu. Microtheca	Mt2	β-tubulin	65°C

5.3.4 Specificity test

Specificity tests with the genus-specific primers for *Diplodia* and *Neofusicoccum* spp. indicate they were specific to their respective target genera (Table 5.2). All Diatrypaceae DNA tested negative to the BD genus-specific primers Dpef1 and Nbt1 primers. Specificity testing for ED pathogen species-specific primers also indicated that they were highly specific to their respective target DNA while all non-target species and the non-template controls tested negative to real-time LAMP (Table 5.2).

Table 5.2. Fungal DNA and synthetic DNA (gBlocks) used for testing the specificity of genus- and species-specific LAMP primer sets using Genie II LAMP assays.

Species	No. of isolates	Amplification for each primer set[a]						
		Dpef1	Nbt1	Cmp1	Elt1	Elp2	Ct2	Mt2
Diatrypaceae genomic DNA								
Cryptovalsa ampelina	3	-	-	+	-	-	-	-
Eutypa lata	3	-	-	-	+	-	-	-
Eutypa leptoplaca	2	-	-	-	-	+	-	-
Eutypella citricola	3	-	-	-	-	-	+	-
Eutypella microtheca	2	-	-	-	-	-	-	+
Diatrypaceae gBlocks								
Cmp1 gBlock	1	-	-	+	-	-	-	-
Elt1 gBlock	1	-	-	-	+	-	-	-
Elp2 gBlock	1	-	-	-	-	+	-	-
Ct2 gBlock	1	-	-	-	-	-	+	-
Mt2 gBlock	1	-	-	-	-	-	-	+
Botryosphaeriaceae genomic DNA								
Diplodia mutila	3	+	-	-	-	-	-	-
Diplodia seriata	3	+	-	-	-	-	-	-
Neofusicoccum australe	3	-	+	-	-	-	-	-
Neofusicoccum luteum	3	-	+	-	-	-	-	-
Neofusicoccum parvum	3	-	+	-	-	-	-	-
Neofusicoccum ribis	2	-	+	-	-	-	-	-
Botryosphaeriaceae gBlocks								
Dip gBlock	1	+	-	-	-	-	-	-
Nbt1 gBlock	1	-	+	-	-	-	-	-

[a] (+) = positive amplification; (-) = no amplification; nt = not tested

5.3.5 Sensitivity test

Sensitivity tests for BD genus-specific primers and ED species-specific primers showed that all assays can detect as low as ~20 fg of synthetic DNA (Table 5.3). All assays can also detect as low as 1-2 pg of fungal DNA alone or in combination with plant DNA. The use of crude plant DNA instead of gDNA has no effect on the amplification of gBlocks but delayed the amplification of fungal gDNA by ~2 minutes for all assays (data not shown).

Table 5.3. Limit of detection for different LAMP assays.

Species (primers)	*gBlocks®	Fungal gDNA	Fungal + plant gDNA
Eutypa dieback LAMP assays			
Cryptovalsa ampelina (Cmp1)	20 fg	1 pg	1 pg
Eutypa lata (Elt1)	20 fg	2 pg	2 pg
Eu. leptoplaca (Elp2)	20 fg	2 pg	2 pg
Eutypella citricola (Ct2)	20 fg	2 pg	2 pg
Eu. microtheca (Mt2)	20 fg	1 pg	1 pg
Botryosphaeria dieback LAMP assays			
Diplodia spp. (Dpef1)	20 fg	1 pg	1 pg
Neofusicoccum spp. (Nbt1)	20 fg	1 pg	1 pg

*gBlocks (Integrated DNA Technologies) – 400-450 bp synthetic DNA used as standards

5.3.6 Efficiency of rapid and low-cost DNA extraction protocol

Of the five protocols tested, the Optigene Plant DNA extraction kit gave consistent positive amplification using real-time LAMP with Tp values of 8-27 minutes. This protocol was easy to use, and all reagents can be stored at ambient temperature. The Lucigen protocol also gave positive amplifications within 10-30 minutes but the reagents need to be stored at -20°C and the method requires sample incubation at 65°C and 98°C, which is not suitable for field use.

The new rapid DNA extraction protocol, referred to as PEG rapid DNA extraction that was adapted from the Optigene rapid plant DNA extraction kit, but using the alkaline PEG lysis buffer developed by Chomczynski and Rymaszewski, (2006), gave consistent amplification for all LAMP assays (Tp values 8 to 27 minutes) similar to the Optigene protocol, but the cost was 80% cheaper than the commercial kit.

5.3.7 PEG Rapid DNA extractions from spore tapes.

The PEG rapid DNA extraction protocol was also suitable for extracting DNA from spore tapes inoculated with *D. seriata* spores but not with *E. lata* ascospores. Real-time LAMP resulted in a signal ratio (number of positive samples out of the total number of reactions) of 3/3 from tape samples inoculated with 14,000, 7,000 and 1,400 *D. seriata* spores and a signal ratio of 2/3 from tapes inoculated with 700 *D. seriata* spores tested positive using Dpef1 primers (Table 5.4).

For spore tapes inoculated with *E. lata* ascospores, real-time LAMP resulted in a signal ratio of 1/3 from tape samples inoculated with 20,000 and 10,000 *E. lata* ascospores, while tapes inoculated with 40,000 and 5,000 spores tested negative to LAMP assays (Table 5.4). Incubation of lysate at 70°C and 98°C or the addition of Proteinase K to the samples did not improve the efficiency of DNA extraction (data not shown).

Table 5.4. LAMP assays to detect *Diplodia seriata* and *Eutypa lata* spores from inoculated tapes using the PEG DNA extraction protocol.

Pathogen (Primer)	No of spores inoculated	LAMP Tp values (min:s)[a]	Signal ratio[b]
Diplodia spp. (Dpef1)	14,000	10:45	3/3
	7,000	11:30	3/3
	1,400	19:30	3/3
	700	23:00	2/3
	Non-template control	-	0/3
Eutypa lata (Elt1)	40,000	-	0/3
	20,000	21:00	1/3
	10,000	21:34	1/3
	5,000	-	0/3
	Non-template control	-	0/3

[a]Time taken to generate a positive result.
[b]Number of positive samples out of the total number of reactions

5.3.8 PEG Rapid DNA extractions from fungal mycelia

The real-time LAMP assays using ED species-specific primers were suitable for rapid identification of fungal isolates (Table 5.5). Of the 14 isolates tested, 10 isolates with *C. ampelina* morphology tested positive to *C. ampelina* LAMP while the remaining four isolates tested negative to the assay. Similarly, the four isolates with *E. lata* morphology further tested positive to *E. lata* LAMP while those with *C. ampelina*-like isolates were negative to the assay. Results indicate the PEG rapid DNA extraction was suitable for fungal mycelia and the two assays were specific to their target species.

Table 5.5. Identification of Diatrypaceae isolates by LAMP assays using crude fungal DNA.

Morphology	Isolates tested positive to LAMP assays (primers)	
	Eutypa lata primers (Elt1)	*Cryptovalsa ampelina* (Cmp1)
Eutypa lata-like (n=4)	4	0
Cryptovalsa ampelina-like (n=10)	0	10

5.3.9 Performance evaluation

5.3.9.1 G-LAMP vs quantitative PCR

G-LAMP assays using gDNA extracted from inoculated wood showed the two BD genus-specific primers Dpef1 and Nbt1 were suitable for detecting low levels of *N. luteum* and *D. seriata* DNA but less sensitive to qPCR. The amount of pathogen DNA for each sample was pre-determined using the existing qPCR assays. The genus-specific primers Dpef1 were able to detect as low as 100 copies of the *D. seriata* DNA from the tested wood samples with an average Tp value of 22 min 45 s, while samples with 30 copies of pathogen DNA tested negative to LAMP. The genus-specific primers Nbt1 further detected 100 copies of *N. luteum* DNA from the wood samples although the Tp value was significantly higher at 27 min 15 s (Table 5.6) compared to the Dpef1 assay.

Table 5.6. Detection of *Diplodia seriata* and *Neofusicoccum luteum* DNA from vacuum-inoculated wood using G-LAMP and qPCR. The number of β-tubulin gene copies for each sample were previously quantified by quantitative PCR prior to real-time LAMP assays using Botryosphaeriaceae multi-species primers.

Pathogen (Primer)	qPCR analysis[a]	G-LAMP[b]
	No. of β-tubulin gene copies/reaction	Tp* values (min:s)[c]
Diplodia spp. (Dp1b)[a]	1,500 copies	15:45
	700 copies	16:15
	400 copies	20:45
	100 copies	22:45
	30 copies	-
	Non-template control	-
Neofusicoccum spp. (Nf1)[a]	75,000 copies	9:30
	46,000 copies	10:15
	3,000 copies	15:00
	100 copies	27:15
	Non-template control	-

[a]Billones-Baaijens *et al*. 2018
[b]G-LAMP – real-time LAMP assays using genomic DNA from inoculated canes.
[c]Time taken to generate a positive result.

5.3.9.2 G-LAMP vs fungal isolations

G-LAMP assays using gDNA from inoculated wood further showed that the assays were more sensitive than fungal isolations (Table 5.7). The *E. lata* specific-primers Elt1 were able to detect *E. lata* from 78% of the inoculated canes. The positive amplifications were observed within 15-25 minutes. This was higher than detection by fungal isolations, which resulted in the pathogen being detected in only 30% of the same canes. The fast-growing *C. ampelina* was also isolated from 100% of the non-inoculated control canes. The assay further detected *E. lata* from 60% of the non-inoculated controls, while this pathogen was not recovered by fungal isolations from the same samples. The assay using *C. ampelina* primers Cmp1 further detected *C. ampelina* DNA from 100% and 60% of the of non-inoculated and inoculated samples, respectively, confirming mixed infections of the two pathogens. All samples were negative to *Eu. citricola* and *Eu. microtheca* (Table 5.7).

Table 5.7. Detection of *Eutypa lata* by fungal isolations and G-LAMP assays from grapevine canes inoculated with *E. lata* ascospores.

Samples	Pathogen detection by fungal isolations		Pathogen detection using *G-LAMP (primers)			
	Eutypa lata re-isolations	*Diatrypaceae* re-isolations	*E. lata* (Elt1)	*C. ampelina* (Cmp1)	*Eu. citricola* (Ct2)	*Eu. microtheca* (Mt2)
Non-inoculated (n=8)	0	100 %	60%	100%	0	0
Inoculated (n=9)	30%	66%	78%	66%	0	0

*G-LAMP – real-time LAMP assays using genomic DNA from inoculated canes.

5.3.9.3 C-LAMP vs fungal isolations

Real-time LAMP assays using crude plant DNA (C-LAMP) from inoculated wood also showed that the assays perform similarly to fungal isolations (Table 1.9). The *E. lata* specific-primers Elt1 were able to detect *E. lata* from 40 (50%) of the inoculated samples tested. Wood samples using the PEG rapid DNA extraction method tested positive to C-LAMP within 15-27 minutes. Of the 40 positive samples, 23 samples have a signal ratio of 2/2 while 17 samples have a signal ratio of 1/2. The LAMP detection was slightly higher than fungal isolations, which resulted in the pathogen being detected in 37 (46%) of the inoculated canes. All non-inoculated controls tested negative to *E. lata* using LAMP and fungal isolations (Table 5.8).

Table 5.8. Detection of *Eutypa lata* by fungal isolations and C-LAMP assays from grapevine canes inoculated with Eutypa lata ascospores.

Samples	*E. lata* detection by fungal isolations	*E. lata* detection using C-LAMP
Non-inoculated (n=30)	0	0
Inoculated (n=80)	37 (46%)	40 (50%)

*C-LAMP – real-time LAMP assays using crude DNA from inoculated canes.

5.3.9.4 Colorimetric LAMP assays

Preliminary tests for BD genus-specific primers Dpef1 (Figure 5.4) and Nbt1 showed that the primers were suitable for colorimetric LAMP. A change in colour from pink to yellow, indicating a positive reaction, was only observed after 60 min incubation while no positive reactions were observed at 30 and 45 min for either primer. All target DNA for Dpef1 and Nbt1 primers tested positive to LAMP while no colour change was observed for the non-target and non-template control samples. The addition of loop primers for each assay speeds up the amplification and the change of colour was observed at 30 min (data not shown).

A change in colour from pink to yellow was further observed for BD colorimetric LAMP using gDNA and crude plant extracts. However, variability between replications was constantly observed between assays (data not shown) when using plant DNA as templates. This variability was not observed when fungal DNA alone was used for the assays. Since colorimetric LAMP is pH dependent, the variability in colour change may be due to the pH of plant DNA used for testing.

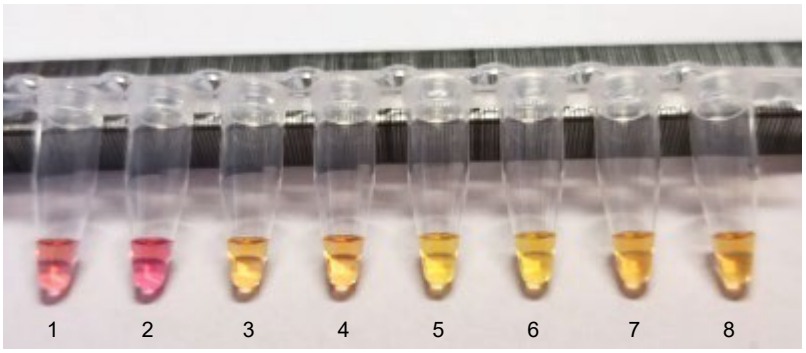


Figure 5.4. Colorimetric LAMP for *Diplodia* genus-specific primers Dpef1. 1) Non-template control; 2) N. parvum B19a – negative control; 3-4) *Diplodia seriata* A142; 5-6) *D. seriata* B135; 7-8) *D. mutila* TV13. Yellow colour indicates a positive reaction while pink colour indicates a negative reaction.

Preliminary tests for *E. lata* species-specific primers Elt1 also showed that the primer was suitable for colorimetric LAMP (Figure 5.5). The change of colour from pink to yellow was observed after 45 min incubation while no positive reactions were observed at 30 min. Higher gBlock concentrations (500 pg to 5 pg) exhibit full yellow colour change while an orange colour was observed for lower concentrations (500 and 50 fg, Figure 5.5). The 60 min incubation resulted in a change of colour for all samples including the non-template and negative control, indicating false positive reactions (data not shown).

The addition of loop primers further speeds up the amplification for each assay and the change of colour was observed at 30 min (data not shown). Similar to the results for BD colorimetric LAMP, variability between replicates was also constantly observed between assays when using plant DNA as a template (data not shown), which may be linked to the pH of the plant DNA. Thus, further optimisation for colorimetric LAMP is essential before it can be used for testing plant materials.

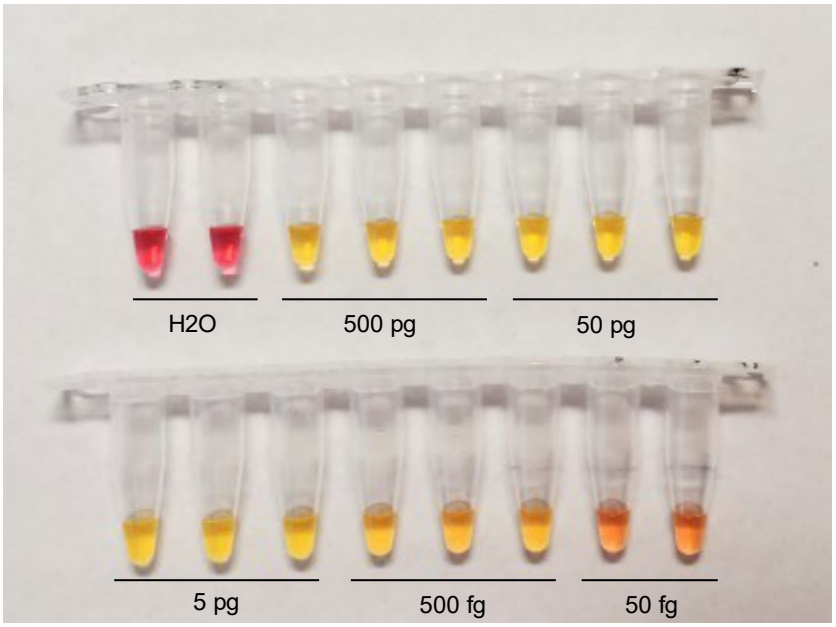


Figure 5.5. Colorimetric LAMP for *Eutypa lata* species-specific primers Elt1 using serially-diluted gBlocks from 500 pg to 50 fg. Pink colour indicates negative reactions, yellow colour indicates positive reactions while orange colour indicates weak positive reactions.

5.4 Discussion

The objective of this study was to develop and evaluate LAMP assays for their suitability and sensitivity to detect the ED and BD pathogens in spore trap samples and infected plant material. These rapid and simple DNA-based methods are aimed at field-based monitoring for ED and BD airborne spores in vineyards and on-site diagnostics of GTD-infected grapevine plant materials. LAMP is a nucleic acid amplification technology that involves a single step process using constant temperatures (Li and Macdonald 2015) and allows testing using simple and portable equipment in the field (Amour *et al.* 2017). LAMP assays have been developed for grapevine pathogens such as *Botrytis cinerea* (Tomlinson *et al.* 2010), the powdery mildew pathogen, *Erysiphe necator* (Thiessen *et al.* 2015), and the downy mildew pathogen, *Plasmopara viticola* (Marimuthu *et al.* 2019), and were found to be useful for rapid detection of these pathogens in vineyards.

In this study, two LAMP genus-specific primers were designed for BD pathogens belonging to the genus *Diplodia* and *Neofusicoccum* and five species-specific primers were designed for ED pathogens, *C. ampelina, E. lata*, *E. leptoplaca, Eu. citricola, and Eu. microtheca*. All seven LAMP primer sets were suitable for real-time LAMP assays using the Genie II instrument (Optigene, UK). Each assay was highly specific and suitable for detecting and discriminating different ED and BD pathogens using gBlocks, genomic DNA and crude DNA extracted from infected plant materials. The Genie II instrument holds two independent control heating blocks that can analyse up to eight samples per block and can detect fluorescence in a single channel (OptiGene 2017). This instrument is lightweight, with an internal rechargeable battery, and is ideal for use outside of the laboratory.

All real-time LAMP assays can detect as little as 1-2 pg of fungal gDNA alone or in combination with plant DNA. The limit of detection (LOD) for the existing qPCR assays developed for SAR 1205 was 200 fg and 20 fg for BD and ED pathogens, respectively. Thus, qPCR is 10 and 100 times more sensitive for detecting BD and ED pathogens, respectively. The LAMP assay results for this study were similar to the LAMP assays developed by Woodhall *et al.* (2019) for GTD pathogens *Phaeomoniella chlamydospora* and *E. lata*.

When tested for their specificity using real-time LAMP, the five species-specific primers designed for the ED pathogens amplified their corresponding target gBlocks, gDNA and gDNA from infected plants while all non-target DNA and non-template controls tested negative to the assays. No cross-reactions were observed when the primer sets were tested using non-target species. The two genus-specific primers for BD pathogens belonging to the genus *Diplodia* (Dpef1) and *Neofusicoccum* spp. (Nbt1),

also amplified their corresponding target species. No cross-reactions were observed when tested against non-target species. All Diatrypaceae DNA tested negative to the two BD genus-specific primers. Similarly, all Botryosphaeriaceae DNA tested negative to all Diatrypaceae LAMP assays.

In this study, a rapid DNA extraction protocol that can be efficiently used to extract crude DNA from plants and other spore tape samples was further developed for field use. The three commercial rapid DNA extractions kits were initially tested and the Optigene protocol gave consistent positive amplifications. This method was also simple and easy to use but is relatively expensive. Thus, a more cost-effective DNA extraction method was developed by combining the Optigene protocol with the alkaline PEG lysis buffer developed by Chomczynski and Rymaszewski, (2006). The PEG rapid DNA extraction method provided consistent positive amplification from inoculated canes similar to the Optigene protocol using real-time LAMP. This method is also easy to use, does not require special storage conditions and can be used in the field but at a lower cost compared to the Optigene commercial kit. A similar in-field DNA extraction method using the PEG/sodium hydroxide buffer (Woodhall *et al.* 2017) was also found suitable for in-field LAMP diagnostics for *Ph. chlamydospora* and *E. lata*.

The PEG rapid DNA extraction protocol was also shown to be suitable to extracting crude DNA from fungal mycelia and inoculated spore tapes. Crude DNA from *E. lata* and *C. ampelina* mycelia were amplified by their corresponding species-specific primers indicating that the real-time LAMP can be used as an alternative method for rapid identification of ED and BD pathogens. For spore tapes, the protocol was found to be efficient for rapid DNA extraction of *D. seriata* spores resulting in positive amplification using real-time LAMP. However, the same protocol gave poor amplification for tapes inoculated with *E. lata* ascospores. Attempts to increase efficiency by incubation at higher temperatures or addition of Proteinase K further resulted in negative amplification by real-time LAMP. The differences in DNA extraction efficiency between the two pathogens may be due to the size of their spores. The ascospores of *E. lata* and other Diatrypaceous species (8-12 × 2-3 µm; Trouillas *et al.* 2011) are less than half the size of BD pathogens (25-27× 7-12 µm, Pitt *et al.* 2010). It is therefore possible that the *E. lata* ascospores are too small to be efficiently removed from the tape surfaces using the rapid extraction protocol, resulting in low DNA yield using the PEG rapid DNA extraction method. Diatrypaceous DNA was efficiently extracted from spore tapes using the method developed Billones-Baaijens *et al.* (2018; SAR 1205). However, this method requires multiple reagents and special equipment such as a centrifuge and incubators making it unsuitable for field use. Thus, further modification to improve DNA extraction efficiency is necessary before the protocol can used for field-based real-time LAMP detection of Diatrypaceous pathogen airborne spores on spore tape. In recent years, spore trap devices that allow automatic detection of airborne spores have been developed (West and Kimber 2015). For instance, the SYield system developed by West *et al.* (2013) can trap *Slerotinia sclerotiorum* spores which are then incubated in semi-selective liquid growth medium and the results of the incubated samples are transmitted wirelessly to a server. Integration of LAMP assays with a spore trapping system such as the Burkard multi-vial cyclone trap can be further explored for future research aimed for real-time and automatic detection of ED and BD spores in the field.

Real-time LAMP assays were further evaluated for their performance relative to qPCR and fungal isolations. For G-LAMP, the two BD genus-specific primers were able to detect low levels of *N. luteum* and *D. seriata* gDNA in vacuum-inoculated vines indicating these assays may be used as a rapid diagnostic tool for infected plant materials. The *E. lata* specific-primers also detected *E. lata* DNA from vineyard canes inoculated with *E. lata* spores in winter 2019, while some samples also tested positive to *C. ampelina*, indicating mixed infections. The detection of *E. lata* DNA from the non-inoculated control canes indicated background infections in the field, and the lower quantity of fungal biomass may explain the lack of *E. lata* detection by isolation on media, compared with inoculated canes. The real-time LAMP assay was at least twice as sensitive at detecting *E. lata* from inoculated wood compared to fungal isolations. However, G-LAMP assays use gDNA for testing, and thus, are only suitable for fully equipped diagnostic laboratories. Genomic DNA extraction is also more costly and time consuming compared to the PEG rapid DNA extraction method.

For C-LAMP, positive detection of *E. lata* from inoculated canes was slightly higher compared to fungal isolations. Thus, C-LAMP assays can potentially be used for the assessment of cane samples from vineyard trials in place of laborious fungal isolations, providing more accurate results. However, 42% of the samples tested had a 1/2 signal ratio, suggesting lower pathogen DNA load in the samples tested. Thus, testing crude samples in duplicates is essential to increase sensitivity of the C-LAMP assays. The G-LAMP was found to be superior to C-LAMP, thus, it is suitable for testing where sensitivity is paramount. However, C-LAMP is a viable alternative for fungal isolations and can be used in limited resource laboratories and on-site testing. Analysing samples with C-LAMP costs five times less than

with G-LAMP. Performance evaluation of C-LAMP for the detection of other ED and BD pathogens from infected plant materials will be incorporated into future research to determine their suitability for onsite testing.

Preliminary tests using the two BD genus-specific primers and the *E. lata* species-specific primer also indicate that these primers were suitable for colorimetric LAMP. A strong colour change was observed for all target DNA after 45 to 60 minutes incubation at 65 to 67°C while no colour change was observed from the non-target controls. Unlike real-time LAMP, colorimetric LAMP does not require expensive equipment like the Genie II and can be performed using simple heating equipment such as heat blocks or water baths. Positive amplification can be assessed visually based on a colour change in the reaction tubes. The change of colour is due to the increase in magnesium pyrophosphate, which is the by-product of amplification (Notomi *et al.* 2000). However, further evaluation of their performance for rapid pathogen detection from infected plant materials gave highly variable results between replicates and assays. The change of colour for colorimetric LAMP is generally linked to the drop in pH that occurs from the extensive DNA polymerase activity in a LAMP reaction, producing a change in solution colour from pink to yellow (https://www.nebiolabs.com.au/tools-and-resources/video-library/loop-mediated- isothermal-amplification-lamp-tutorial). The variability in the colour change between replicates may be linked to the PEG lysis buffer used for rapid DNA extraction that is highly alkaline (pH of 13.5). Further studies to identify rapid DNA extractions suitable for colorimetric LAMP are essential before this can be used for ED and BD field-based diagnostics.

Current diagnostics for ED for BD rely primarily on fungal isolations and PCR-based techniques that are time-consuming and labour intensive or require expensive equipment and highly skilled staff to perform the analysis. PCR-based diagnostics also rely on an inhibitor-free DNA source. Thus, rapid and simple LAMP assays designed for on-site diagnostics of infected grapevine plant materials can allow testing for ED and BD pathogens in limited resource environments. In this study, we developed real-time LAMP assays using the Genie II instrument to detect ED and BD pathogens in infected plant materials, spore tapes and fungal mycelia. A low cost and simple DNA extraction protocol was also developed for LAMP and was found highly suitable for rapid DNA extraction from infected wood and other substrates. All seven real-time LAMP assays were highly specific and suitable for detecting and discriminating different ED and BD pathogens. The LAMP assays were at least twice as sensitive as conventional fungal isolation but were less sensitive than existing qPCR methods. This may offer cost effective, simple and robust diagnostic tools in the field or limited resource environments. These assays can be further recommended for diagnostic laboratories and nurseries for the rapid detection of ED and BD pathogens from infected plant materials. The species-specific primers designed for the ED pathogens were also suitable for rapid identification of Diatrypaceae isolates and can be potentially used as an alternative method for DNA sequencing for isolate identification. Future research aims to further validate LAMP assays in the field. This will lead to the establishment of protocols for commercial diagnostic laboratories that will be made available for industry-wide testing.

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6 INOCULUM DISPERSAL

6.1 Burkard volumetric spore trapping

6.1.1 Introduction

Eutypa dieback (ED) and Botryosphaeria dieback (BD) are considered the two most important grapevine trunk diseases in Australia, causing significant yield reduction and threatening the sustainability of Australian vineyards. Spores of the causal fungal pathogens are released from fruiting bodies embedded in diseased woody parts of vines as well as in pruning debris left in the vineyard (van Niekerk *et al*. 2010). These spores are dispersed primarily by rain splash, travel distances depending on the wind speed (Úrbez-Torres *et al*. 2010; Baskarathevan *et al*. 2013), and infect pruning wounds resulting in cankers, dieback and eventually death of vines. Thus, understanding the spore dispersal patterns of these pathogens will help determine the critical times of the year when spores are abundant in Australian vineyards and when vines are at greatest risk of infection.

Between 2013 and 2016, a study was undertaken to investigate the spore dispersal patterns of ED and BD pathogens in four wine growing regions in Australia (Sosnowski *et al*. 2017; SAR 1205). Burkard volumetric spore traps were deployed in the Barossa Valley and Coonawarra, South Australia (SA) and in the Hunter Valley and Griffith, New South Wales (NSW). Spore trap tapes were collected and replaced monthly at each site and analysed using two quantitative PCR (qPCR) assays (Sosnowski *et al*. 2017; Billones-Baaijens 2018; SAR 1205) to detect Diatrypaceae and Botryosphaeriaceae spores. The two qPCR protocols developed using multi-species primers in this study accurately detected and quantified up to nine Diatrypaceae or Botryosphaeriaceae species in a single sample. Preliminary results showed dispersal of ED and BD pathogen spores was sporadic and varied between regions, season and year. Spores were generally recorded during or immediately after rain, but not all rain events resulted in spore detection. Since the results from the three-year study indicate high variability in spore release patterns between four regions, the sampling area was expanded to other climatic regions of Australia in 2017 to further provide localised data for each region.

The objective of this study was to determine the presence of, and to quantify the GTD pathogen spores, in relation to climate throughout the year in selected climatic regions of Australia, using rapid and highly sensitive DNA-based molecular tools. This report presents the main outcome of the three-year spore trapping studies in the four wine growing regions in Australia conducted as part of the project SAR 1205. The data from the six spore traps that were deployed from 2017-2021 across Australian wine regions as part of the project SAR 1701-1.1 are also presented. Data were analysed to determine the critical times of the year when GTD pathogen spores are abundant in vineyards located in different climatic regions. This will assist growers in making decisions on optimal timing of pruning and wound treatment for GTDs.

6.1.2 Methods

6.1.2.1 Spore trapping

For the spore trapping conducted in SAR 1205, two volumetric spore traps (Burkard Manufacturing Co, Rickmansworth, England) based on a design by Hirst (1952), were fitted with a battery and solar panel for recharging (Measurement Engineering Australia) and deployed in the Barossa Valley and Coonawarra, SA between August 2013 to December 2016 (Figure 6.1a-b). Two additional spore traps were deployed in Griffith and the Hunter Valley, NSW between April 2014 to November 2016 and May 2014 to October 2016, respectively (Figure 6.1c-d). For SAR 1701-1.1, only the Barossa Valley and Coonawarra spore traps were continued from SAR 1205, and following the purchase of two new Burkard volumetric traps, three were deployed in the Adelaide Hills, Clare and McLaren Vale, SA in January to March 2017, and one trap was deployed in Tumbarumba, NSW in June 2017.

For spore trapping, Melinex tapes (2×33 cm) were mounted in a Burkard spore drum and coated with a petroleum-based adhesive, Tangle-Trap® (The Tanglefoot® Company, USA) using a paintbrush. The drum was placed inside the spore trap chamber and the clock was set for 36 days. The Coonawarra and Tumbarumba traps were placed at the end of a vineyard row, but on 18 October 2018, the Coonawarra trap was relocated 700 m from its original position to be located within 5 m of the vineyard (Figure 6.1e-f). The Clare and McLaren Vale spore traps were placed within 5 m of vines (Figure 6.1g-h), while the Adelaide Hills spore trap was placed approximately 10 m from vines (Figure 6.1i), and the

Barossa Valley spore trap was located about 20 m from vines (Figure 6.1a). All spore traps were operated with the impact orifice 0.5 m above the ground with an airflow of 10 litres per minute. Spore drums were collected and replaced monthly at each site and sent to the laboratory where tapes were removed from the drum (Figure 6.1j), cut longitudinally into two pieces (Figure 6.1k). One piece was cut further into two-day sections (1 × 1.9 cm) using the 36-day rotation cutting block (Burkard Manufacturing Company; Figure 6.1l). The remaining section was stored in electrical conduit at room temperature in a sealed box with moisture absorbing silica gel (BDH, England; SA samples) and at -20ºC (NSW samples). Each two-day tape section was placed in a sterile 1.5 mL Eppendorf tube (lysing tube) containing ~300 mg of (400-600 µm diameter) Ballatoni beads (Daintree Scientific, Australia) and one 1/4" ceramic sphere (MP Biomedicals) and stored at room temperature until required for DNA extractions. Total DNA was extracted from each tape using the methods developed by Billones-Baaijens *et al.* (2018).

All DNA extracted from spore trap tapes from all regions was analysed by nested-PCR and multi-species qPCR assays developed for ED and BD for SAR 1205 (Billones-Baaijens *et al.* 2018, Sosnowski *et al.* 2017). All spore trap DNA samples were initially tested for the presence or absence of both ED and BD spores by nested-PCR followed by qPCR for ED and BD pathogens using the methods described in Sosnowski *et al.* 2017 (SAR 1205).

The calculated number of ED and BD spores in two-day spore tape samples was plotted with the total rainfall and mean minimum and maximum temperatures for the same period for each location to determine the correlation between spore release and weather data. A computer modeller was consulted to further investigate the relationship between spore release and climatic factors (relative humidity, dew point and wet bulb temperatures and wind speed) using data from SAR 1205. Weather data for SA sites were sourced from the Bureau of Meteorology (www.bom.gov.au). The weather data for Tumbarumba, NSW were sourced from the NSW DPI weather station located approximately 100 m from the spore trap (https://www.dpi.nsw.gov.au/agriculture/horticulture/ grapes/weather-stations-network/wsn). The weather data for Coonawarra were obtained from AWS 26091, located approximately 900 metres from the spore trap; the Barossa Valley from AWS 23373, which was co-located with the spore trap; the Clare Valley from a private AWS co-located with the spore trap; the McLaren Vale from a private AWS located 6 km from the spore trap; and the Adelaide Hills from Balhannah, located approximately 3 km from the spore trap.

6.1.2.2 Identification of Diatrypaceous spores from vineyards

To confirm the identity of the Diatrypaceous spores detected by qPCR, 30 spore tape samples with high amplification (Cq values <20) were randomly selected from different sample dates from each region and used for Sanger DNA sequencing. To generate the required amount of amplicons essential for sequencing, a nested-PCR was performed by initially amplifying 2 µL of spore tape DNA extracts using the universal primer ITS1 and ITS-4 (White *et al.* 1990) with the annealing temperature of 55°C at 25 cycles. The second step PCR was conducted by running 1 µL of the Step 1 PCR products as templates using the primers DITS-1F and DITS-1R and the protocol previously described in SAR 1205 (Sosnowski *et al*. 2017). PCR products from the second step of each nested PCR were visualised by electrophoresis following the protocol as previously described.

All products were purified using the Favorgen® PCR purification kit (Favorgen Biotec Corp, Taiwan) following the manufacturer's protocols. All purified products were quantified using fluorometry (Quantus, Promega, USA) and 10-12 ng of purified DNA were sent to the Australian Genome Research Facilities (AGRF, Sydney, NSW) for Sanger sequencing using the DITS1F primer. The resulting sequences and chromatograms were analysed using the DNAMAN 5.2 (Lynnon Biosoft©) and Chromas Lite 2.1© (Technelysium PTY Ltd) software. All trimmed DNA sequences were subsequently identified using the Basic Local Alignment Search Tool (BLAST) in GenBank (https://www.ncbi.nlm.nih.gov/genbank). The species was considered a match if it had 100% homology with a continuous section of at least 270 bp of the ITS sequence of the reference species.

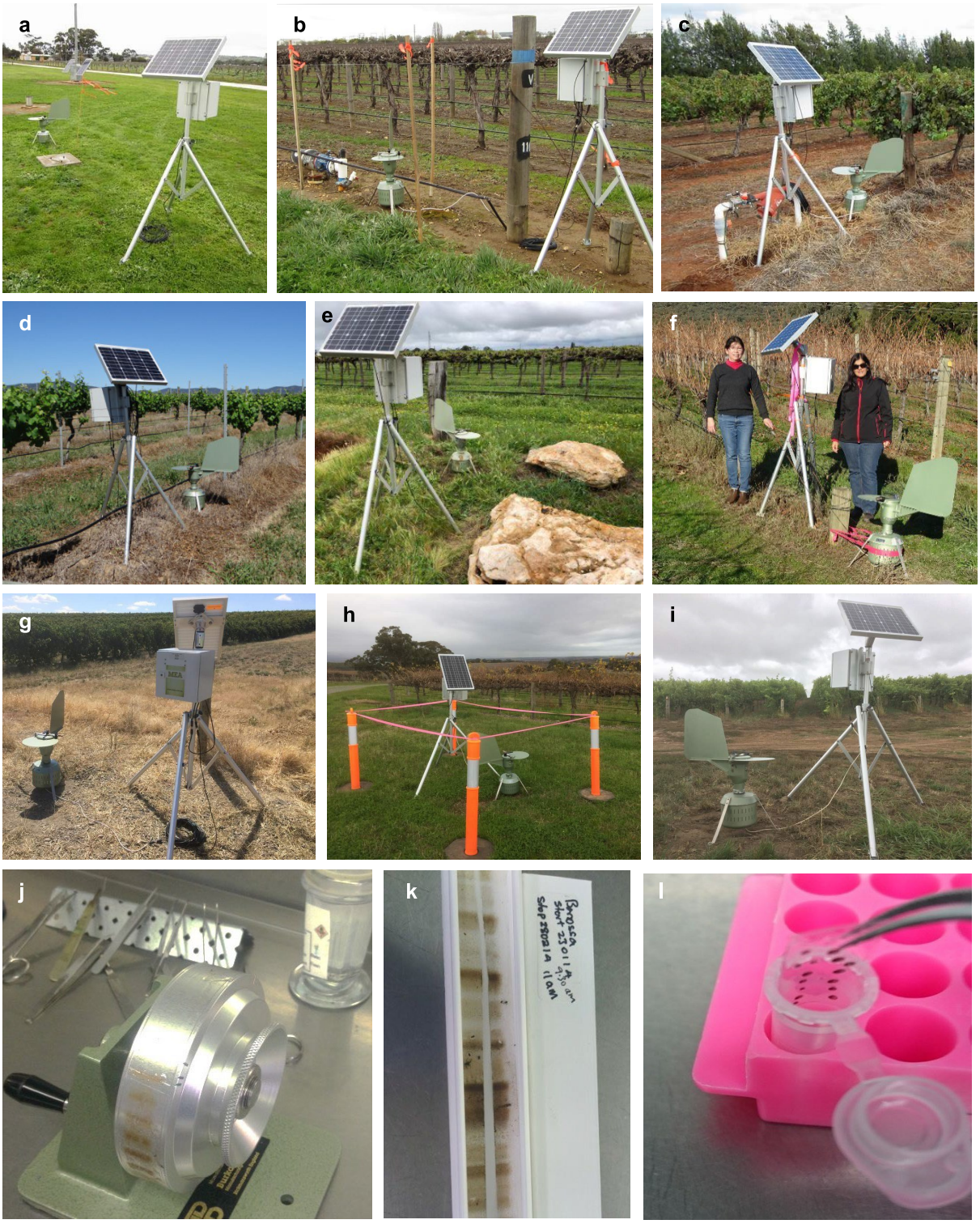


Figure 6.1. Burkard spore traps used for spore-trapping studies and placed within vineyards from 2013-2016 in South Australia, Barossa Valley (a) and Coonawarra (b) and in New South Wales, Griffith (c) and Hunter Valley (d); and from 2017-2020, relocated in Coonawarra (e) and new locations of Tumbarumba NSW (f), along with Clare Valley (g), McLaren Vale (h) and Adelaide Hills (i) in SA. A spore drum mounted with Melinex tape (j); longitudinally cut spore tape stored in electrical conduit (k) and spore tape section placed in a tube for DNA extraction (l).

6.1.3 Results

6.1.3.1 Spore trapping

2013-2016 (SAR 1205)

A total of 1,931 spore tape samples collected over three to four seasons for each site were analysed and 792 (41%) tested positive to either or both ED and BD pathogens. The qPCR showed that the spores of ED and BD pathogens were released sporadically at different times of the year in all four regions. The spore numbers and frequency of detection varied between regions, pathogens and years. For ED pathogens, the incidence of spore release was highest in the Hunter Valley, NSW at 34%, while the incidence of spore release for ED pathogens for the other three regions was 21 (Coonawarra and Barossa) to 25% (Griffith) (Table 6.1). For BD pathogens, the incidence of spore release was highest in Coonawarra, SA at 23% and lowest in the Barossa Valley, SA at 9%. Occasionally, both BD and ED pathogen spores were trapped in the same period and released continuously for several days. The spore release usually occurred during or after rain, with as little as 0.2 mm of rain resulting in spore release of either ED or BD pathogens. However, occasionally spores were released more than a week after a rainfall event.

Table 6.1. Total number of two-day spore tape samples collected from the four wine growing regions and percentage of samples testing positive to ED and BD pathogens for 2013-2016 (SAR 1205).

Region	Total samples	Positive to BD pathogens	No. of BD pathogen spores	Positive to ED pathogens	No. of ED pathogen spores
Barossa Valley, SA	606	54 (9%)	8 - 1,320	130 (21%)	30 - 3,483
Coonawarra, SA	493	115 (23%)	8 - 6,182	104 (21%)	30 - 10,608
Griffith, NSW	407	61 (15%)	8 - 921	102 (25%)	30 - 12,450
Hunter Valley, NSW	425	81 (19%)	8 - 333	145 (34%)	30 - 30,162

From the Barossa Valley, analyses of the 606 spore tape samples collected from September 2013 to December 2016, 130 (21%) and 54 (9%) of the two-day samples tested positive to ED and BD pathogens, respectively (Table 6.1, Figure 6.2). For ED pathogens, the incidence of detection was greater in 2015 and 2016 at 28% and 37%, respectively, compared to 2013 and 2014 at 6% and 5%, respectively. In 2015-2016, the number of ED pathogen spores trapped was relatively high on several occasions with nine samples having more than 1,000 spores trapped over a two-day period and with the greatest number (3,483) trapped in January 2016. In contrast, the incidence of BD pathogen detection was greater in 2014 at 20% compared to the other years (6 - 9%). The number of BD pathogen spores per two-day sample ranged from 8 - 1320 spores with the highest number trapped in September 2013. In 2016, the total annual rainfall was 738 mm, which was significantly higher compared to 480 and 348 mm for 2014 and 2015, respectively.

From the Coonawarra, analyses of the 493 spore tape samples collected from August 2013 to December 2016, 104 (21%) and 115 (23%) of the samples tested positive to ED and BD pathogens, respectively (Table 6.1, Figure 6.3). For ED pathogens, the incidence of detection was greater in 2016 at 36% compared to 15% in 2015. For BD pathogens, the incidence of detection was also greater in 2016 at 37% compared to 2015 at 19%. In 2016, the number of ED and BD pathogen spores detected was relatively high on a few occasions with two and three spore tape samples having more than 5,000 BD or ED pathogen spores trapped over a two-day period, respectively, with the greatest number (10,608 ED pathogen spores) trapped in March 2016. The number of BD pathogen spores per two-day sample ranged from 8 to 6,182 spores with the highest number trapped in July 2016. In 2016, the total annual rainfall was 768 mm, which was significantly higher compared to 449 mm for 2015. The number of samples for 2013 was low since the spore trapping commenced in August 2013. In 2014, the spore trap malfunctioned for seven months and further reduced the number of samples collected during this period.

From Griffith, analyses of the 407 spore tape samples collected from June 2014 to November 2016, 102 (25%) and 61 (15%) of the samples tested positive to ED and BD pathogens, respectively (Table

6.1, Figure 6.4). For ED pathogens, the incidence of spore detection was also greater in 2016 at 32% compared to 2013 and 2014 at 22% for both years. In 2014, the number of ED pathogen spores detected was high with 10 samples having more than 1,000 spores trapped over a two-day period with the greatest number (12,450) trapped in April 2014. In contrast, the incidence of BD pathogen spore detection was greater in 2014 at 20% compared to 14% and 10% in 2015 and 2016, respectively. The number of BD pathogen spores per two-day sample ranged from 8 - 428 spores with the highest number trapped in January 2015. The spore trap malfunctioned for four months in winter 2016 resulting in a lower number of samples collected. In 2016, the total annual rainfall was 686 mm, which was higher compared to 346 and 435 mm for 2014 and 2015, respectively.

From the Hunter Valley, analyses of the 425 samples collected from June 2014 to November 2016, 145 (34%) and 81 (19%) of the samples tested positive to ED and BD pathogens (Table 6.1, Figure 6.5). For ED pathogens, spore detection was greater in 2016 at 56% compared to 2014 and 2015 at 19% and 25%, respectively. In 2016, the number of ED pathogen spores detected was extremely high (up to 30,100) with nine two-day samples having more than 7,000 spores and the greatest number detected in January-May 2016. For BD pathogens, spore detection was also greater in 2016 at 34% compared to 17% for both 2014 and 2015. The number of BD pathogen spores per two-day sample ranged from 8 - 227 with the greatest number trapped in December 2014. The highest annual rainfall was recorded in 2015 at 1,006 mm, compared to 662 and 761 mm for 2014 and 2016, respectively.

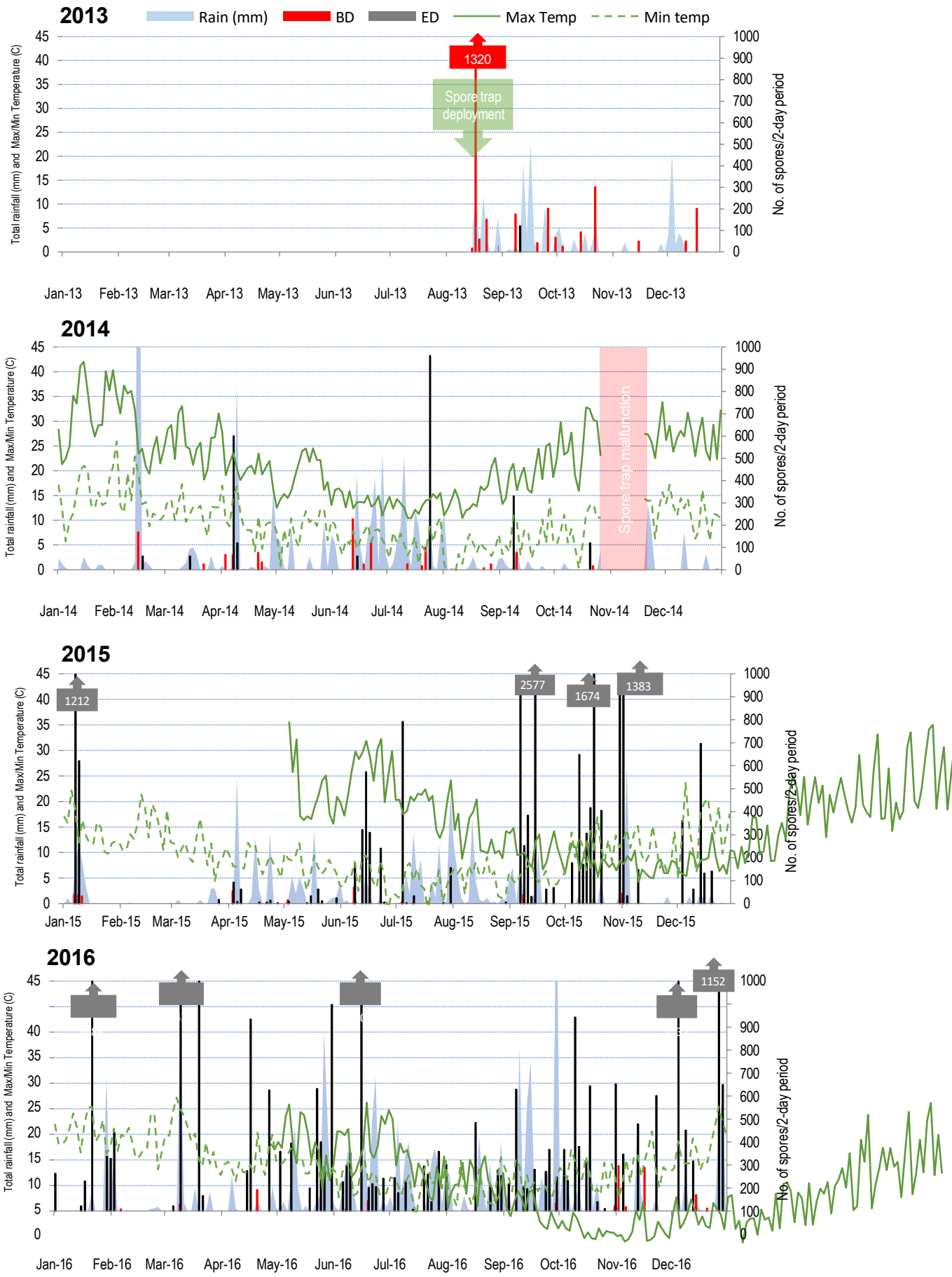
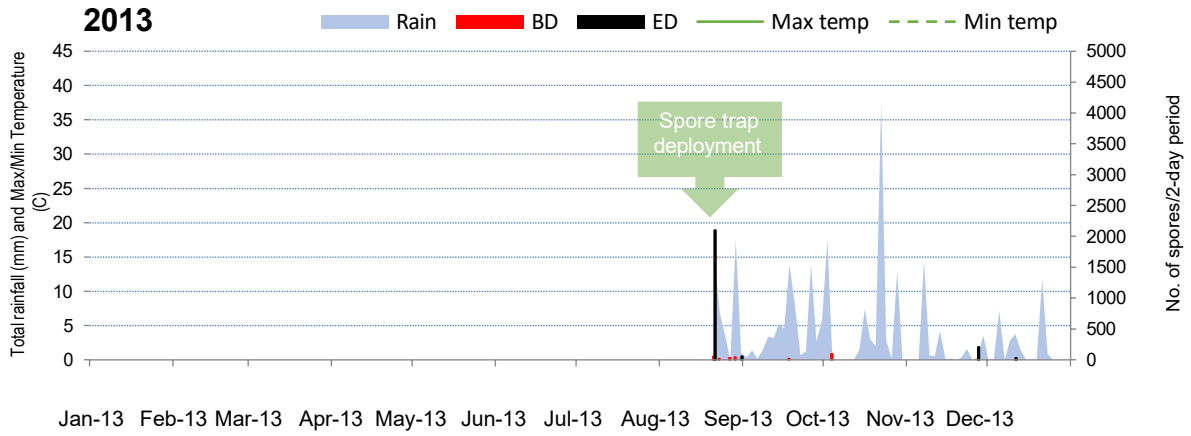
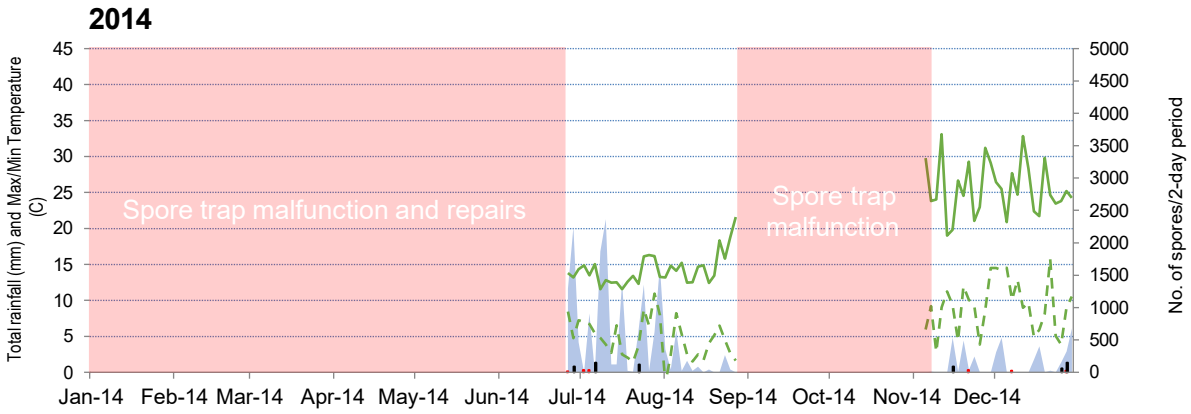
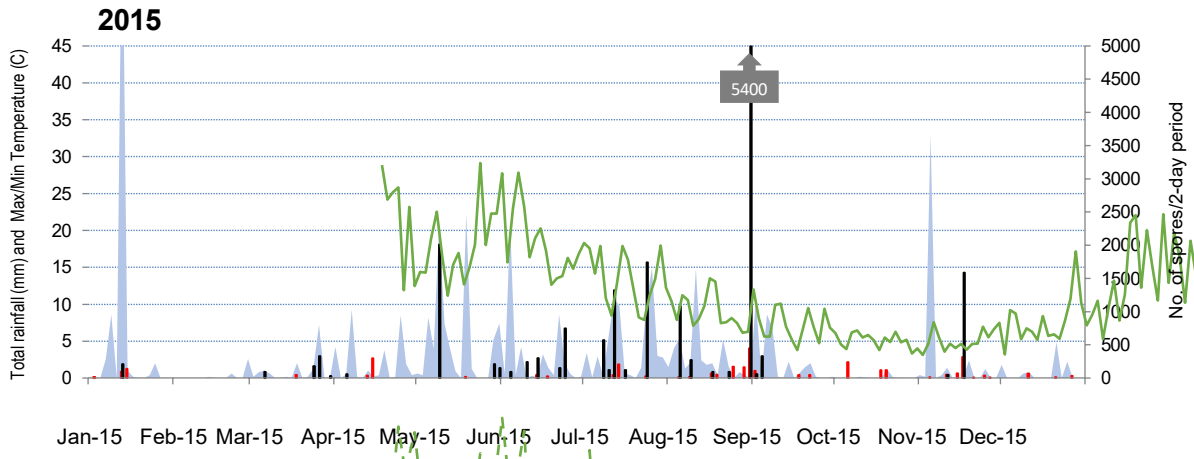


Figure 6.2. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in the Barossa Valley, South Australia, from September 2013 to December 2016, plotted

against rainfall and maximum temperature (www.bom.gov.au).


2013
Rain
BD
ED
Max temp
Min temp
Spore trap deployment
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
Jan-13 Feb-13 Mar-13 Apr-13 May-13 Jun-13 Jul-13 Aug-13 Sep-13 Oct-13 Nov-13 Dec-13


2014
Spore trap malfunction and repairs
Spore trap malfunction
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
Jan-14 Feb-14 Mar-14 Apr-14 May-14 Jun-14 Jul-14 Aug-14 Sep-14 Oct-14 Nov-14 Dec-14


2015
5400
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
Jan-15 Feb-15 Mar-15 Apr-15 May-15 Jun-15 Jul-15 Aug-15 Sep-15 Oct-15 Nov-15 Dec-15

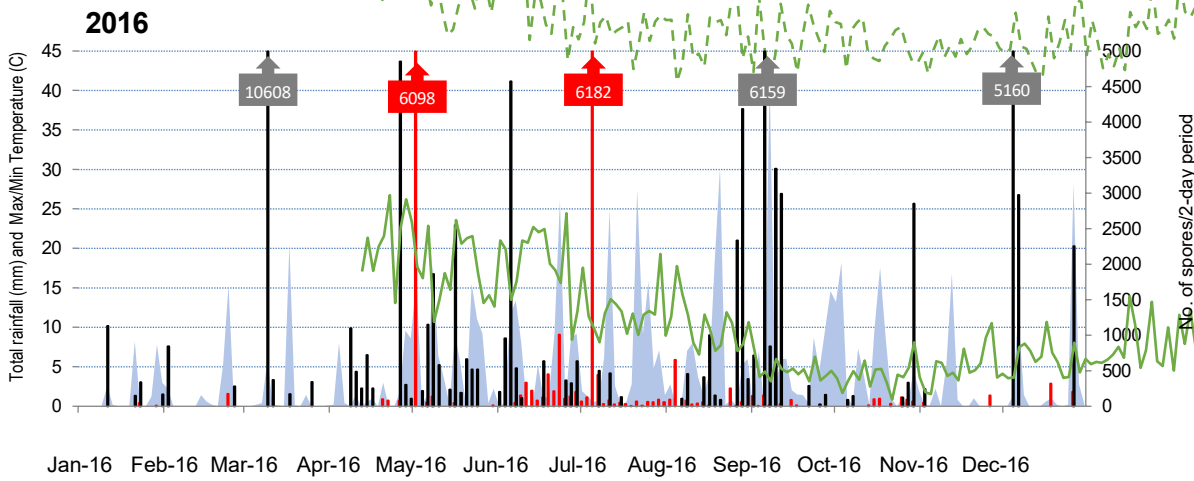

2016
10608
6098
6182
6159
5160
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
Jan-16 Feb-16 Mar-16 Apr-16 May-16 Jun-16 Jul-16 Aug-16 Sep-16 Oct-16 Nov-16 Dec-16

Figure 6.3. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogens spores detected per two-day period in Coonawarra, South Australia, from September 2013 to December 2016 and plotted against rainfall and maximum temperature (www.bom.gov.au).

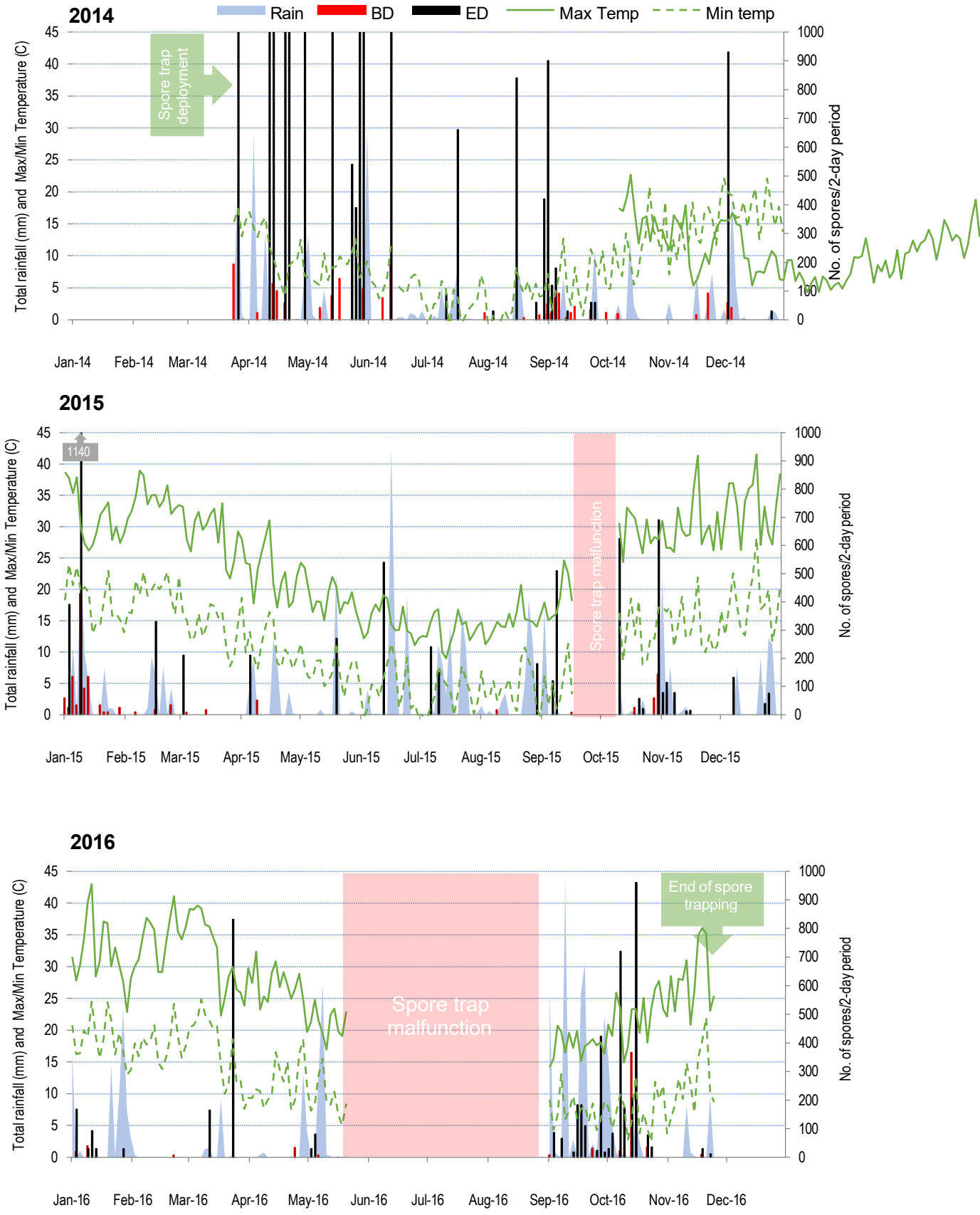


Figure 6.4. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in Griffith, New South Wales, from April 2014 to November 2016 and plotted against rainfall and maximum temperature (www.bom.gov.au).

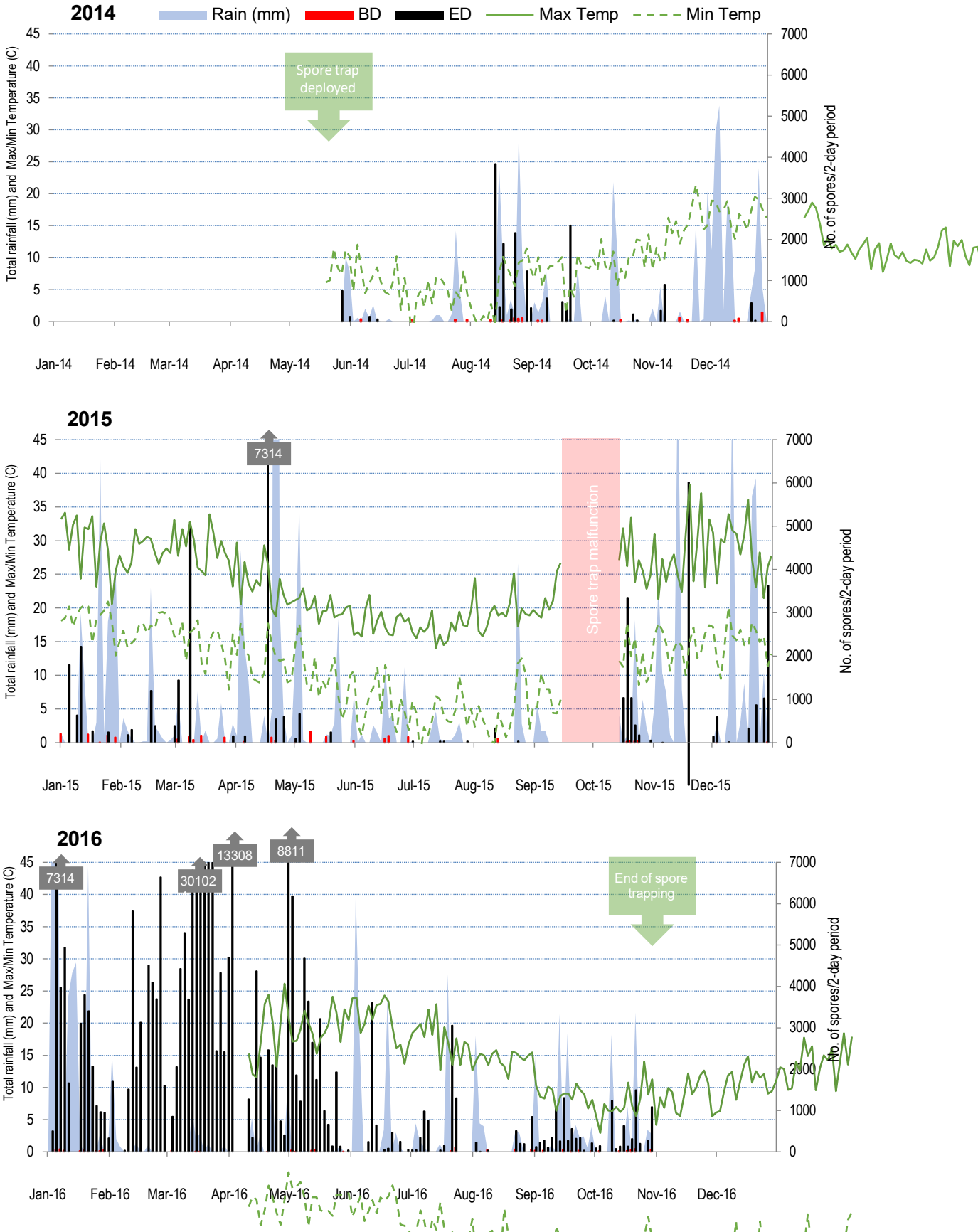


Figure 6.5. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in the Hunter Valley, New South Wales, from June 2014 to November 2016 and plotted against rainfall and maximum temperature (www.bom.gov.au).

2017-2021

A total of 5,071 spore tape samples collected in 2017-2021 across the six sites were analysed and 2,541 (50%) tested positive to either or both ED and BD pathogens (Table 6.2). Similar to the results in SAR 1205, qPCR data showed spores of ED and BD pathogens were detected sporadically at different times of the year with the spore numbers and frequency of detection varying between regions, pathogens and years. For ED pathogens, the overall incidence of spore detection was highest in the Adelaide Hills at 43% and lowest in Clare, SA at 22%. For BD pathogens, the overall incidence of detection was highest in Tumbarumba, NSW at 24% and lowest in the Adelaide Hills, SA at 15%. Occasionally, both BD and ED pathogen spores were detected in the same period and then continuously for several days. The spore detection usually occurred during or after the occurrence of rain, with as little as 0.2 mm of rain resulting in detection of either ED or BD pathogen spores.

Table 6.2. Total number of two-day spore tape samples collected from the four wine growing regions and percentage of samples testing positive to ED and BD pathogens from 2017-2021.

Region	Total samples	Positive to BD	No. of BD spores	% positive to ED	No. of ED spores
Adelaide Hills, SA	865	128 (15%)	8-2,903	369 (43%)	30-14,542
Barossa Valley, SA	897	140 (16%)	8-479	222 (25%)	30-6,500
Clare, SA	837	131 (16%)	8-346	188 (22%)	30-7455
Coonawarra, SA	865	144 (17%)	8-3,045	351 (41%)	30-25,298
McLaren Vale, SA	836	161 (19%)	8-637	222 (27%)	30-17,599
Tumbarumba, NSW	771	185 (24%)	8-7,796	300 (39%)	30-25,478

From the Adelaide Hills, analyses of the 865 spore tape samples collected from March 2017 to December 2021, 369 (43%) and 128 (15%) of the samples tested positive to ED and BD pathogens, respectively (Table 6.2, Figure 6.6). For ED pathogens, the incidence of spore detection was greater in 2021 at 59% compared to other seasons (33-42%). For BD pathogens, the incidence of spore detection was greater in 2020 at 25% compared to other seasons (9-15%). The number of ED spores per two-day sample ranged from 30 to 14,543 with the highest number trapped in June-July 2019. The number of BD pathogen spores per two-day sample ranged from 8 - 2,903 with the highest number detected in April 2020. The highest annual rainfall was recorded in 2017 and 2020 at 888 and 833 mm, respectively, while the lowest rainfall was recorded in 2019 at 261 mm.

From the Barossa Valley, analyses of the 897 spore tape samples collected from January 2017 to December 2021, 222 (25%) and 140 (16%) of the samples tested positive to ED and BD pathogens, respectively (Table 6.2, Figure 6.7). For ED pathogens, the incidence of spore detection was also greater in 2021 at 39% compared to other seasons at 15-23%. For BD pathogens, the incidence of detection was similar in 2017, 2018 and 2021 at 16%-19%, and slightly higher compared to 2019 and 2020 at 10% and 13%, respectively. The number of ED pathogen spores per two-day sample ranged from 30 to 6,500 with the highest number detected in September 2020. The number of BD pathogen spores per sample ranged from 8 - 479 spores with the highest number detected in November 2021. The highest total annual rainfall was recorded in 2017, 2020 and 2021 at 490, 456 and 473 mm, respectively which were significantly higher compared to 320 and 291 mm for 2018 and 2019, respectively.

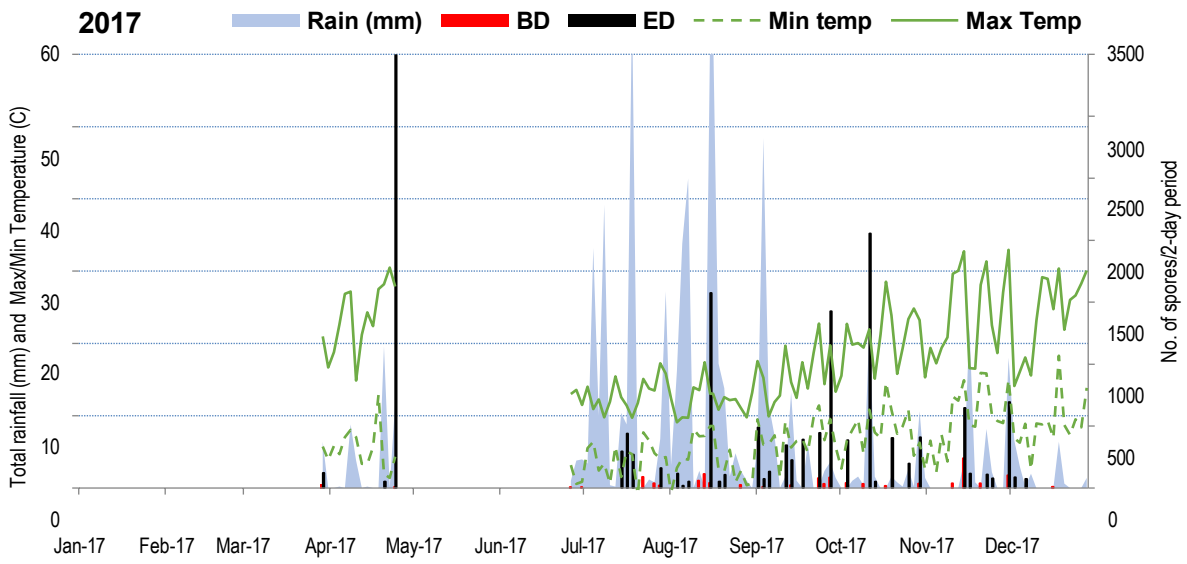
From the Clare Valley, analyses of the 837 spore tape samples collected from March 2017 to December 2021, 188 (22%) and 131 (16%) of the samples tested positive to ED and BD pathogens, respectively (Table 6.2, Figure 6.8). For ED pathogens, the incidence of detection was significantly greater in 2021 at 59% compared to other seasons (10-16%). The number of ED pathogen spores detected was extremely high in 2021 (up to 7,455) with seven two-day samples having more than 1,000 spores and the greatest number detected between August and November 2021. For BD pathogens, the incidence of detection was similar during the five seasons at 15 to 18%. The number of BD pathogen spores per two-day sample ranged from 8 to 346 spores with the highest number trapped in November 2019. The annual rainfall was higher in 2020 and 2021 at 414 and 392 mm, respectively while the 2019 annual rainfall was the lowest in five seasons at 130 mm.

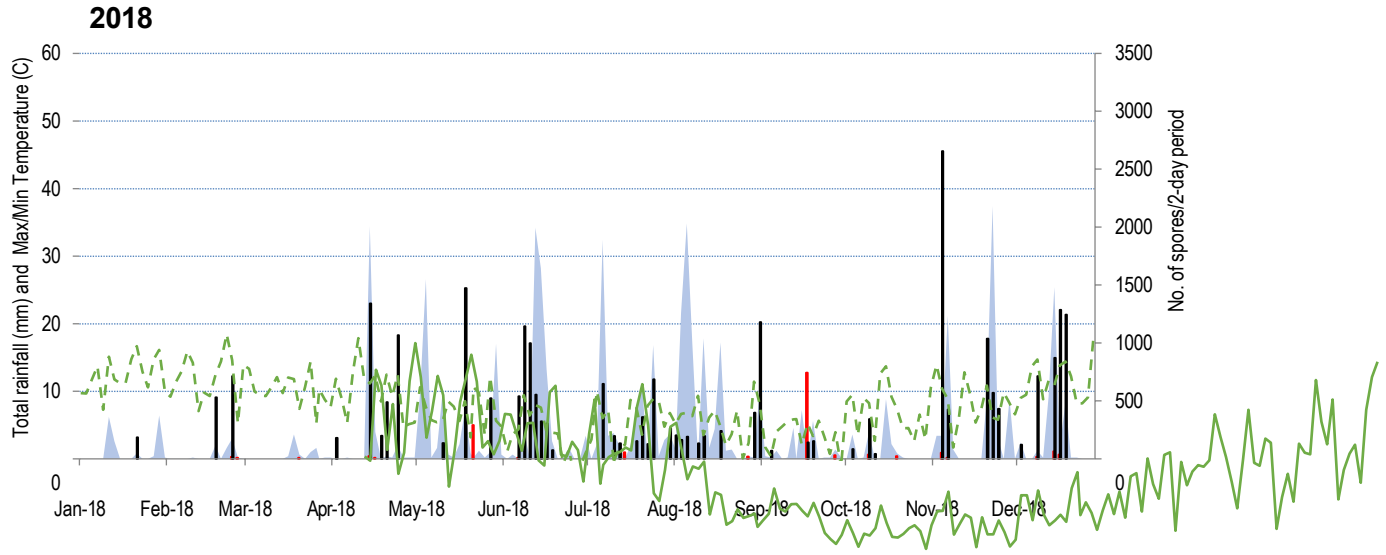
From the Coonawarra, analyses of the 865 spore tape samples collected from January 2017 to December 2021, 351 (41%) and 144 (17%) of the samples tested positive to ED and BD pathogens,

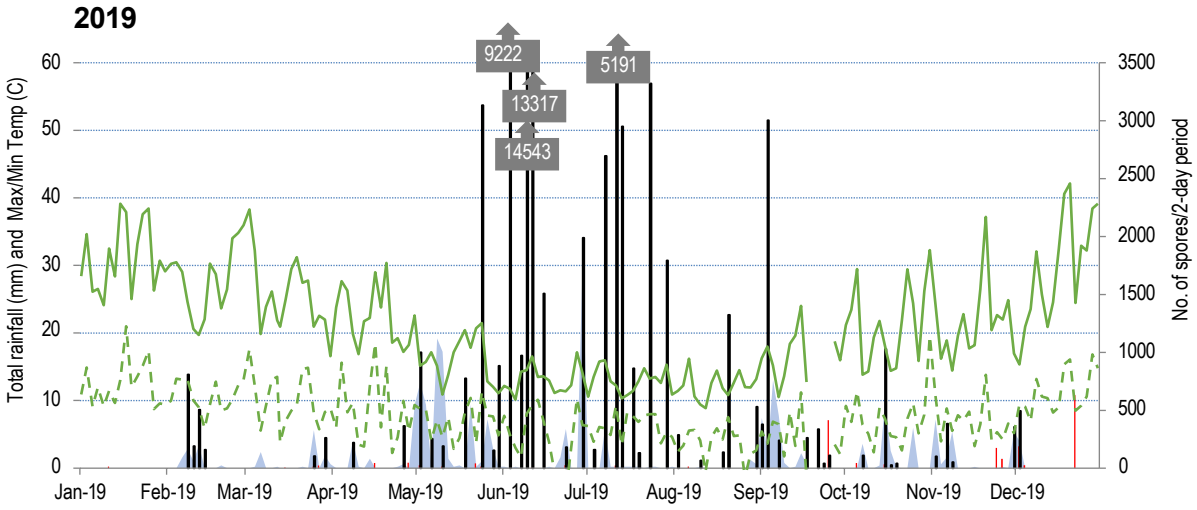
respectively (Table 6.2, Figure 6.9). For ED pathogens, the incidence of detection was greater in 2020 and 2021 at 47 and 59% compared to the other three seasons (28-38%). For BD pathogens, the incidence of detection was similar during the five seasons at 14 to 24%. The number of ED pathogen spores detected per two-day sample was extremely high in 2020 (up to 25,298) with five samples having more than 5,000 spores and the greatest number detected between June and October. The number of BD pathogen spores per two-day sample ranged from 8 to 3,045 spores with the highest number detected in May 2017. The total annual rainfall for 2017 and 2020 were 649 and 583 mm, respectively and significantly higher compared to the other three seasons (358-466 mm).

From McLaren Vale, analyses of the 836 spore tape samples collected from April 2017 to December 2021, 222 (27%) and 161 (19%) of the samples tested positive to ED and BD pathogen spores, respectively (Table 6.2, Figure 6.10). For ED pathogens, the incidence of detection was greater in 2017 at 39% compared to the other seasons 20-31%. The highest number of spores per two-day sample (17,599) was trapped in January 2020. However, the number of ED pathogen spores trapped was also extremely high in 2017 (up to 7,989) with six two-day samples having more than 3,000 spores and the greatest number detected between August and September 2017. For BD pathogens, the incidence of detection was lower in 2020 at 12% compared to the other seasons 20-31%. The number of BD pathogen spores per two-day sample ranged from 8 to 637 spores with the highest number detected in November 2017. The total annual rainfall for 2020 and 2021 was 668 and 619 mm, respectively, which were higher compared to the other seasons (362-510 mm).

From Tumbarumba, analyses of the 771 samples collected from June 2017 to December 2021, 300 (39%) and 185 (24%) of the samples tested positive to ED and BD pathogens, respectively (Table 6.2, Figure 6.11). For ED pathogens, the incidence of detection was greater in 2021 at 54% compared to the other four seasons at 32 to 36%. In 2020, the spore trap malfunctioned on three occasions, resulting in a reduced number of samples collected during the season. The number of ED pathogen spores trapped per two-day sample was extremely high in 2019-2021 (up to 25,478) with 17 samples having more than 5,000 spores and the greatest number detected between April and July 2019. For BD pathogens, the incidence of detection was higher in 2021 at 31% compared to the other four seasons at 21 - 23%. The incidence of detection was particularly high between September to November 2021. The number of BD pathogen spores per two-day sample ranged from 8 - 7,396 with the greatest number detected in April 2019. The annual rainfall for 2021 was extremely high at 1362 mm compared to the other seasons 663-805 mm.


2017
Rain (mm)
BD
ED
Min temp
Max Temp
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
0
10
20
30
40
50
60
0
500
1000
1500
2000
2500
3000
3500
Jan-17
Feb-17
Mar-17
Apr-17
May-17
Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17


2018
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
0
10
20
30
40
50
60
0
500
1000
1500
2000
2500
3000
3500
Jan-18
Feb-18
Mar-18
Apr-18
May-18
Jun-18
Jul-18
Aug-18
Sep-18
Oct-18
Nov-18
Dec-18


2019
9222
13317
14543
5191
Total rainfall (mm) and Max/Min Temp (C)
No. of spores/2-day period
0
10
20
30
40
50
60
0
500
1000
1500
2000
2500
3000
3500
Jan-19
Feb-19
Mar-19
Apr-19
May-19
Jun-19
Jul-19
Aug-19
Sep-19
Oct-19
Nov-19
Dec-19

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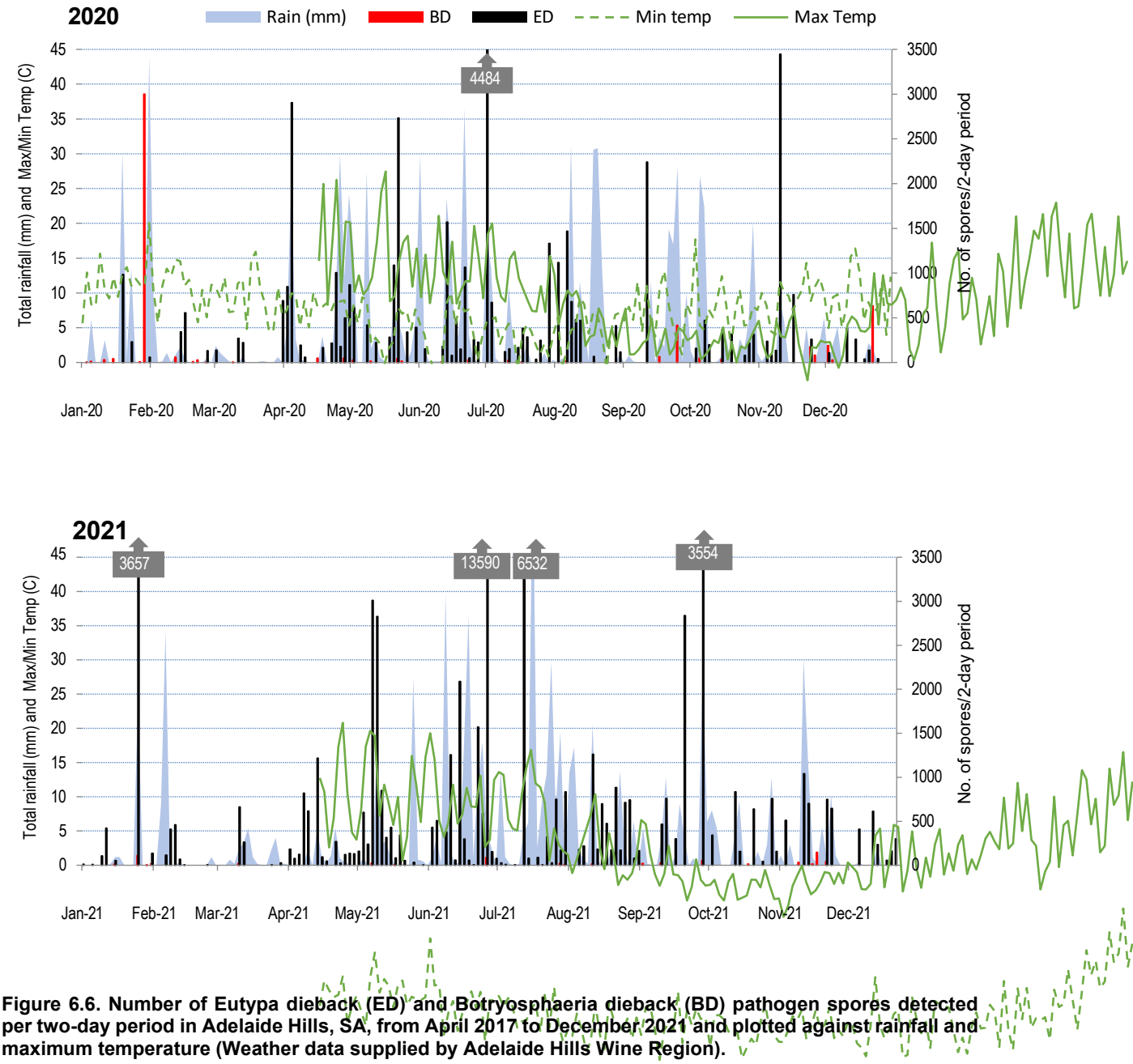
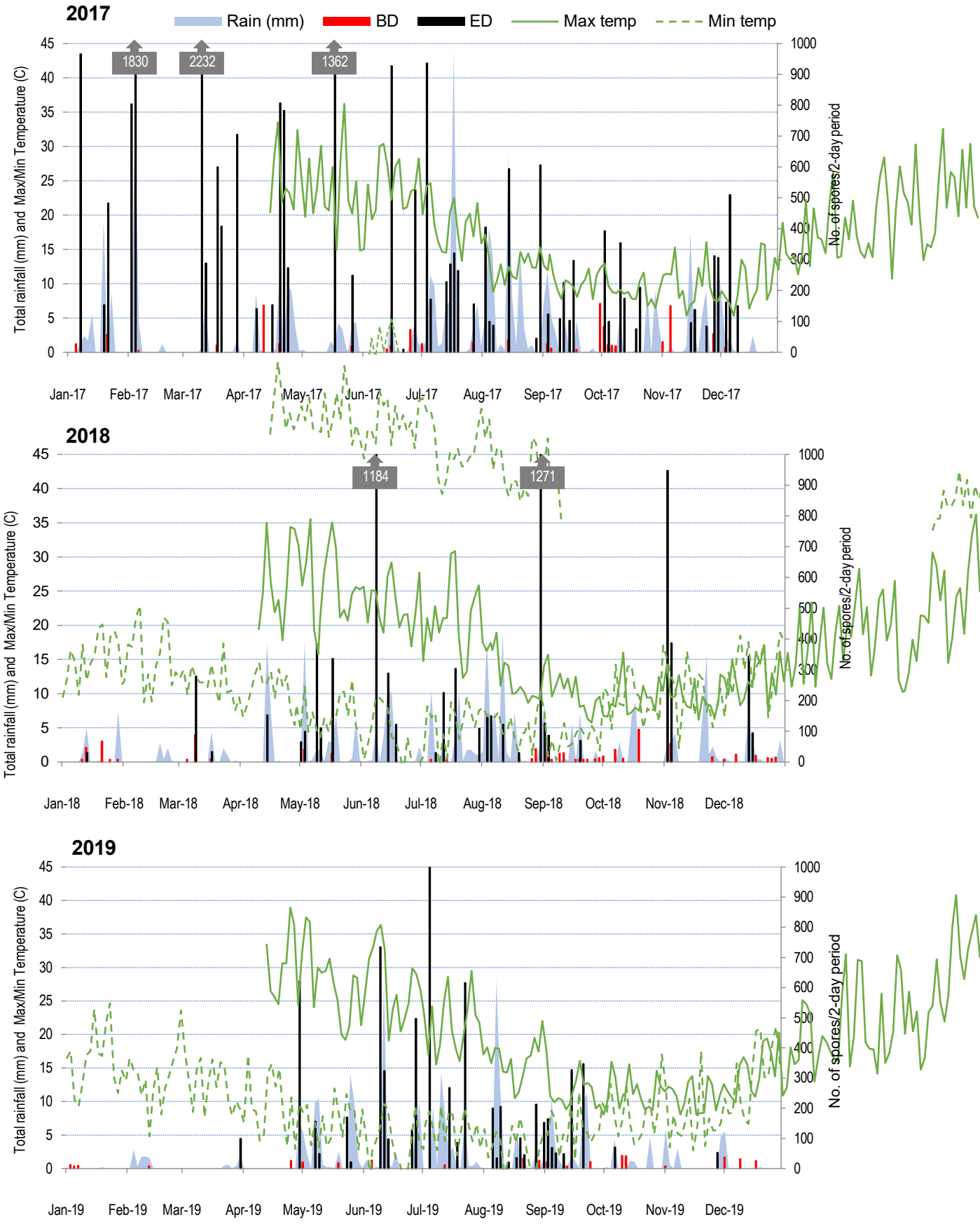


Figure 6.6. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in Adelaide Hills, SA, from April 2017 to December 2021 and plotted against rainfall and maximum temperature (Weather data supplied by Adelaide Hills Wine Region).



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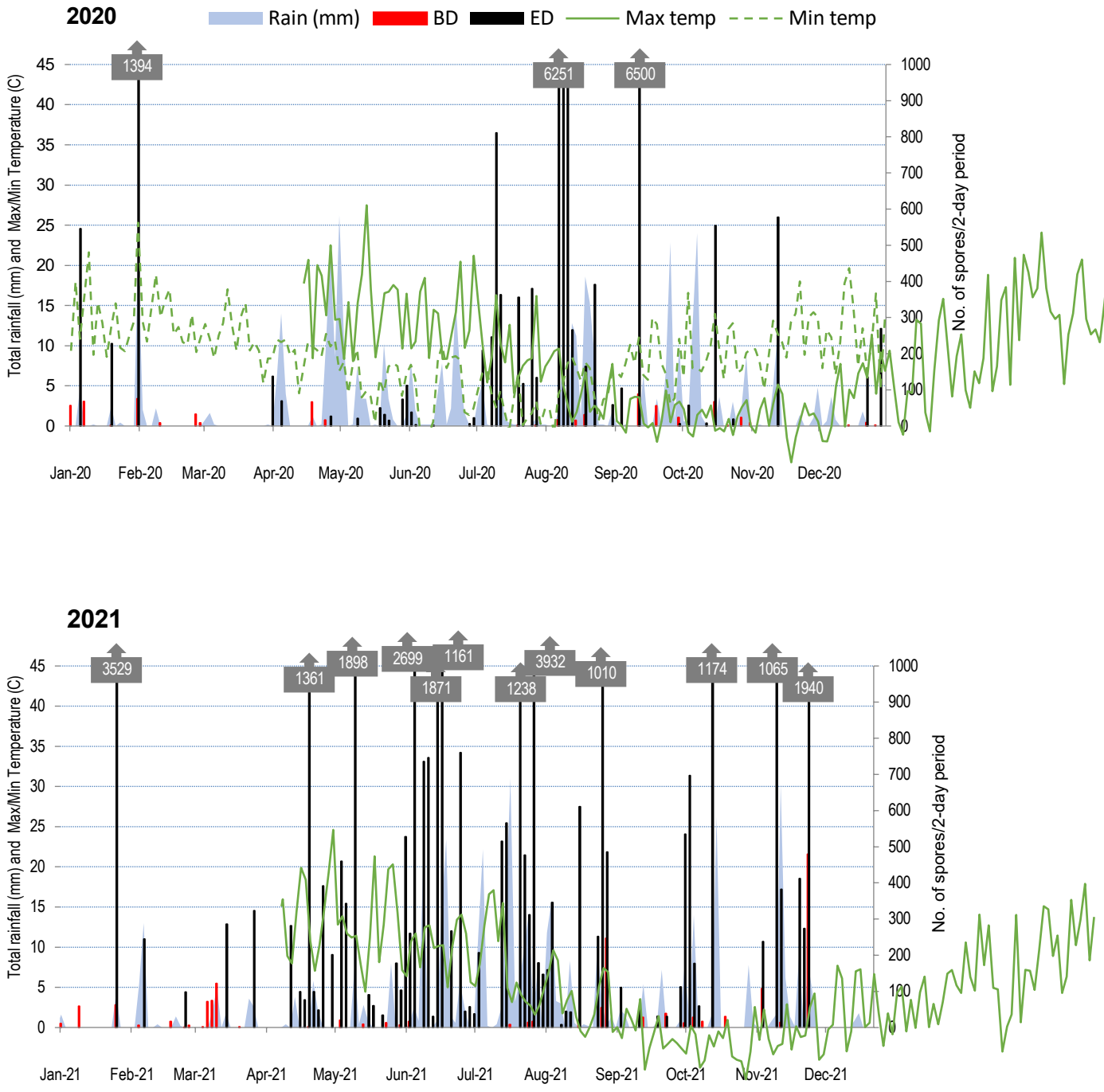
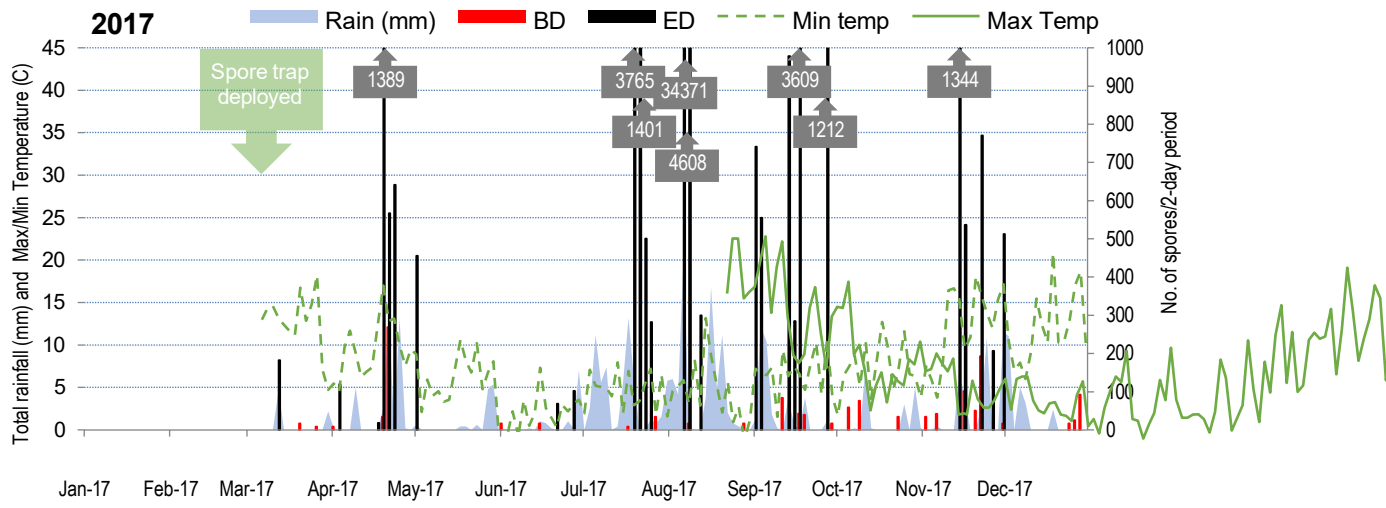
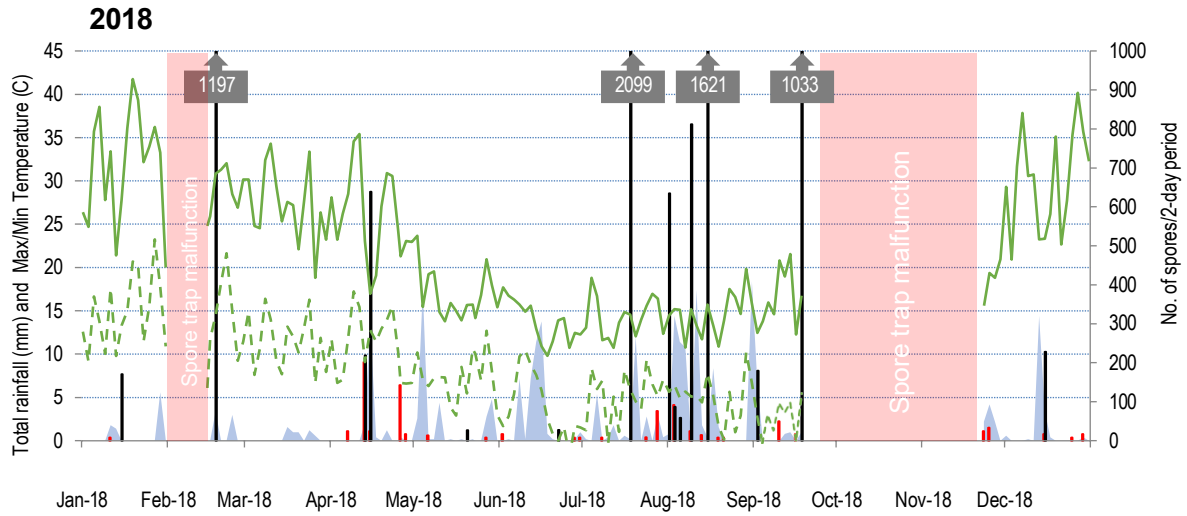
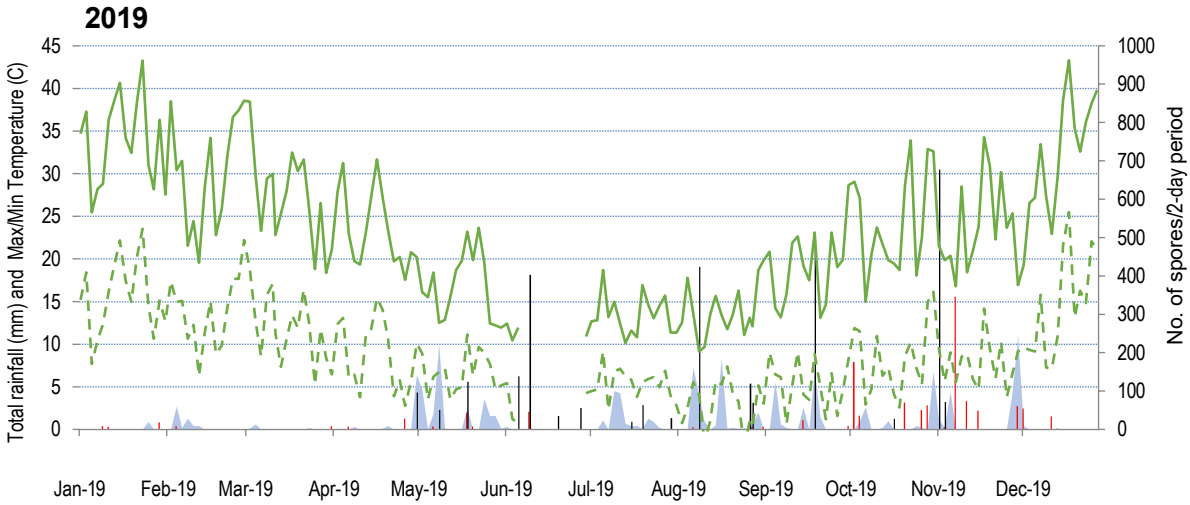


Figure 6.7. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in the Barossa Valley, SA, from January 2017 to December 2021 and plotted against rainfall and maximum temperature (www.bom.gov.au).







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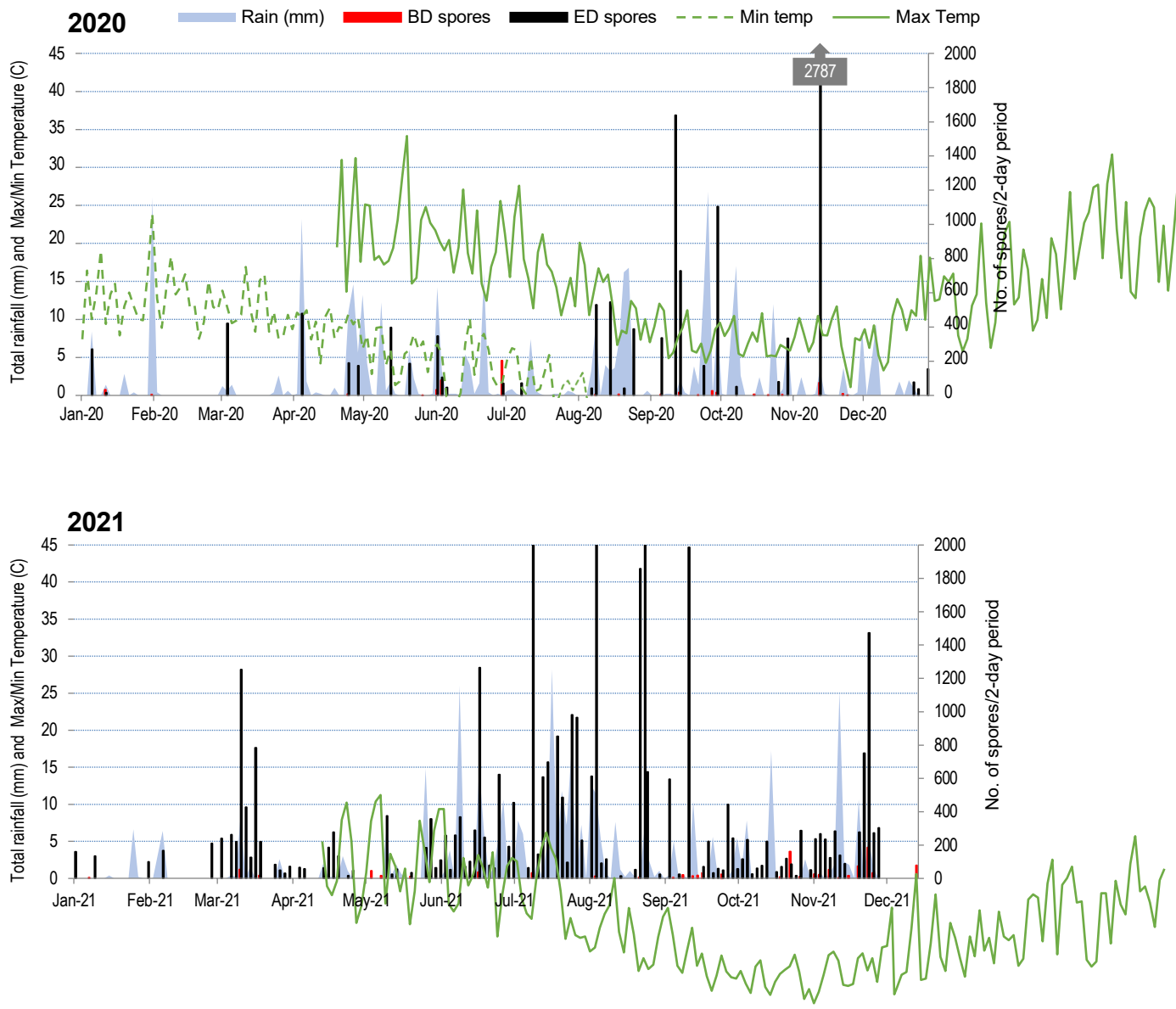
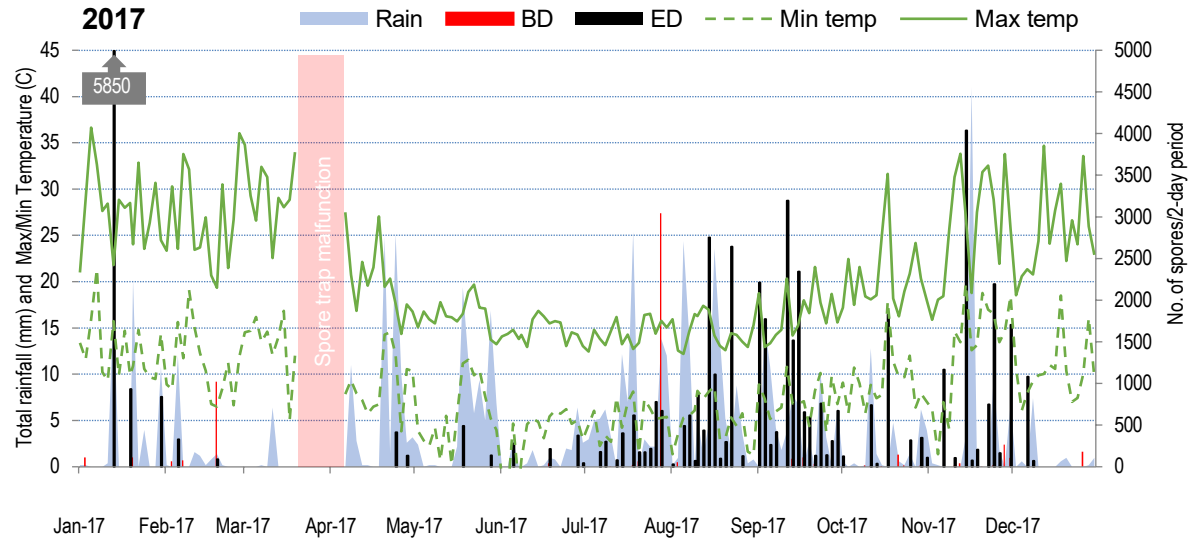
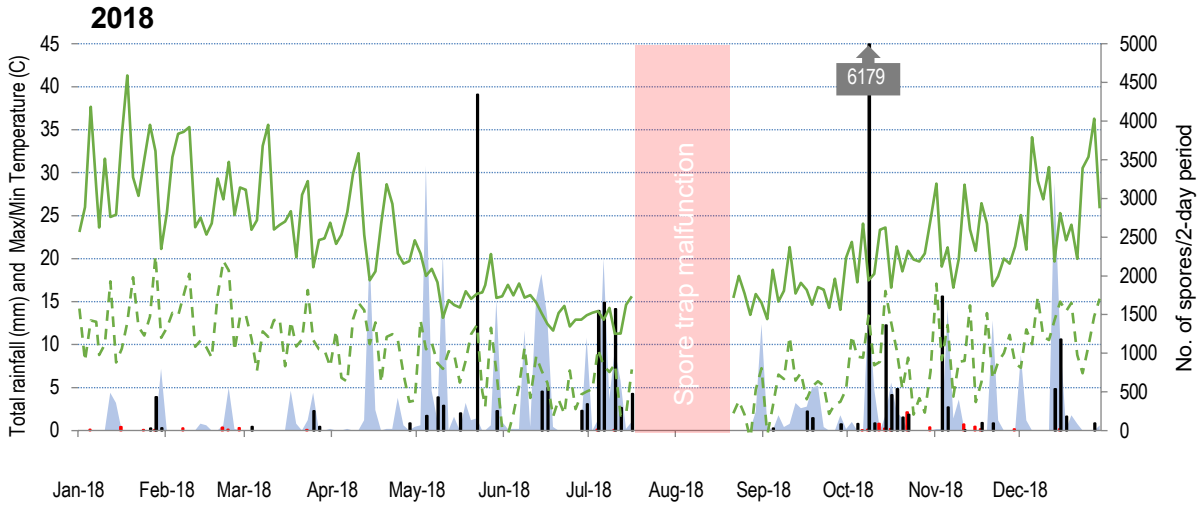
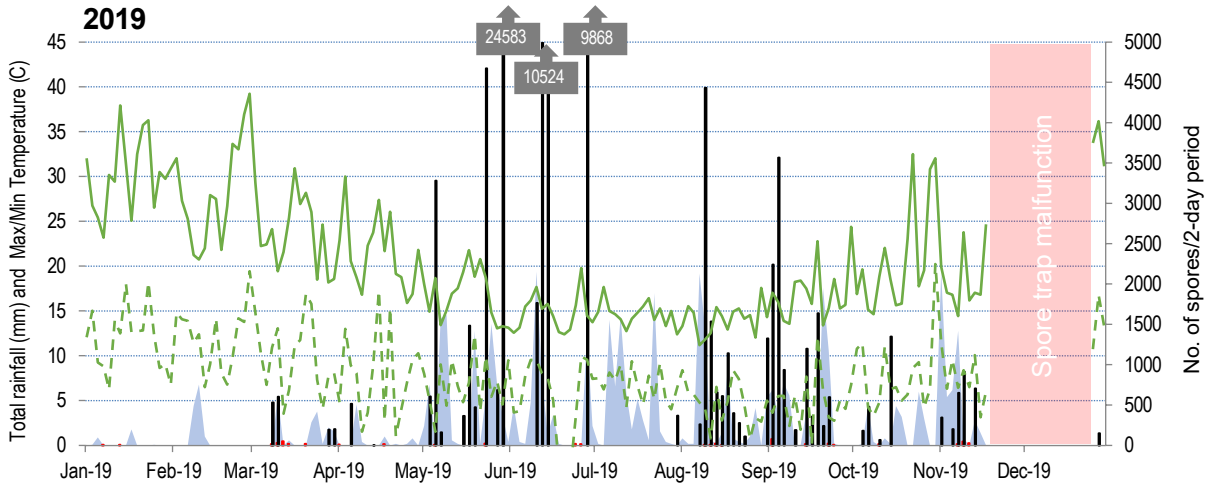


Figure 6.8. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in the Clare Valley, SA, from March 2017 to December 2021 and plotted against rainfall and maximum temperature.







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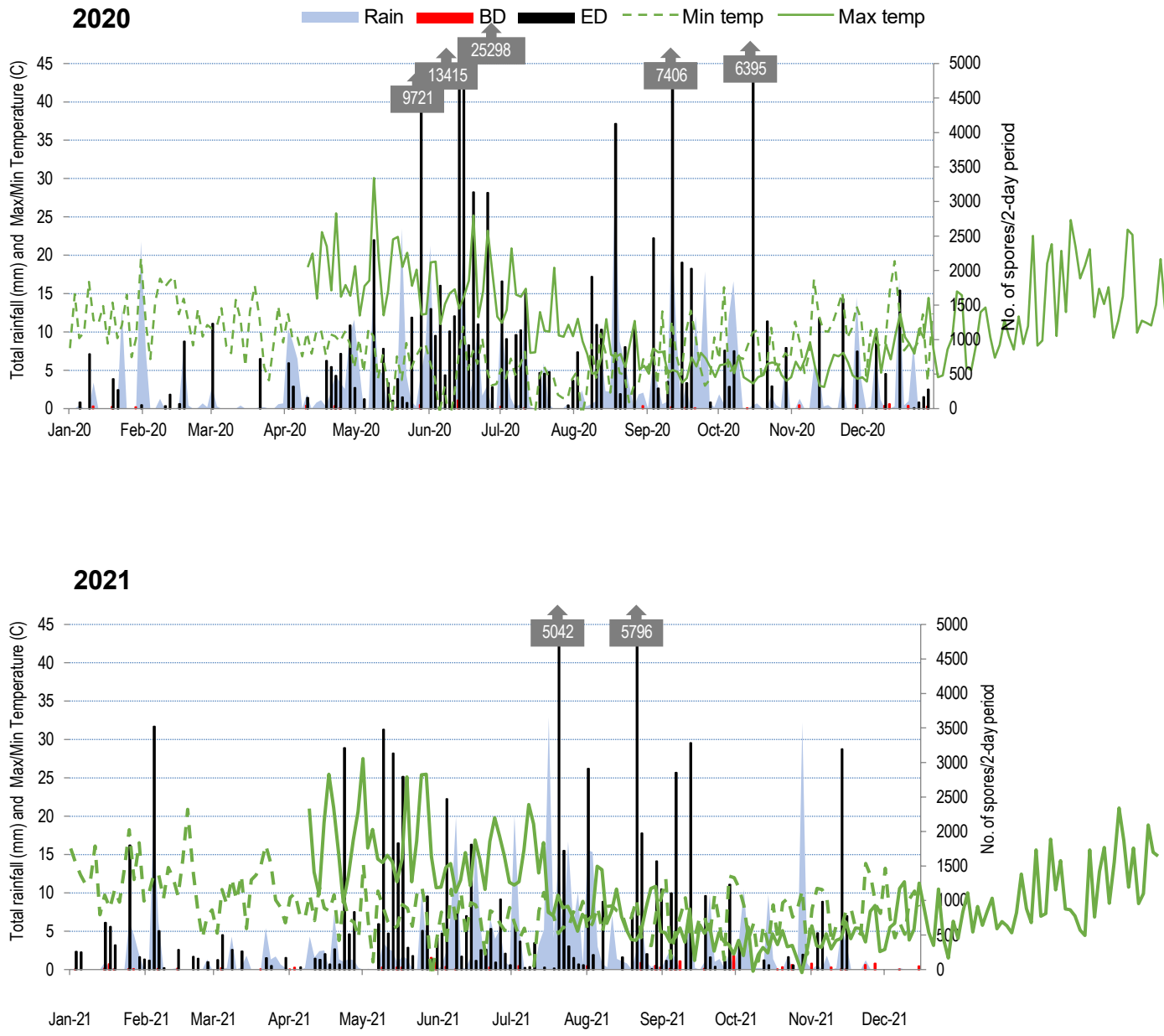
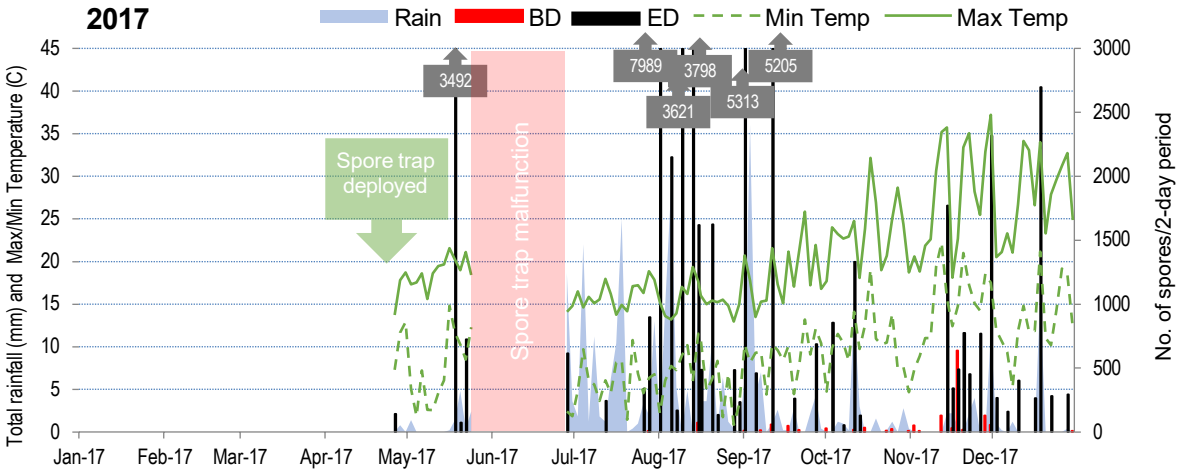
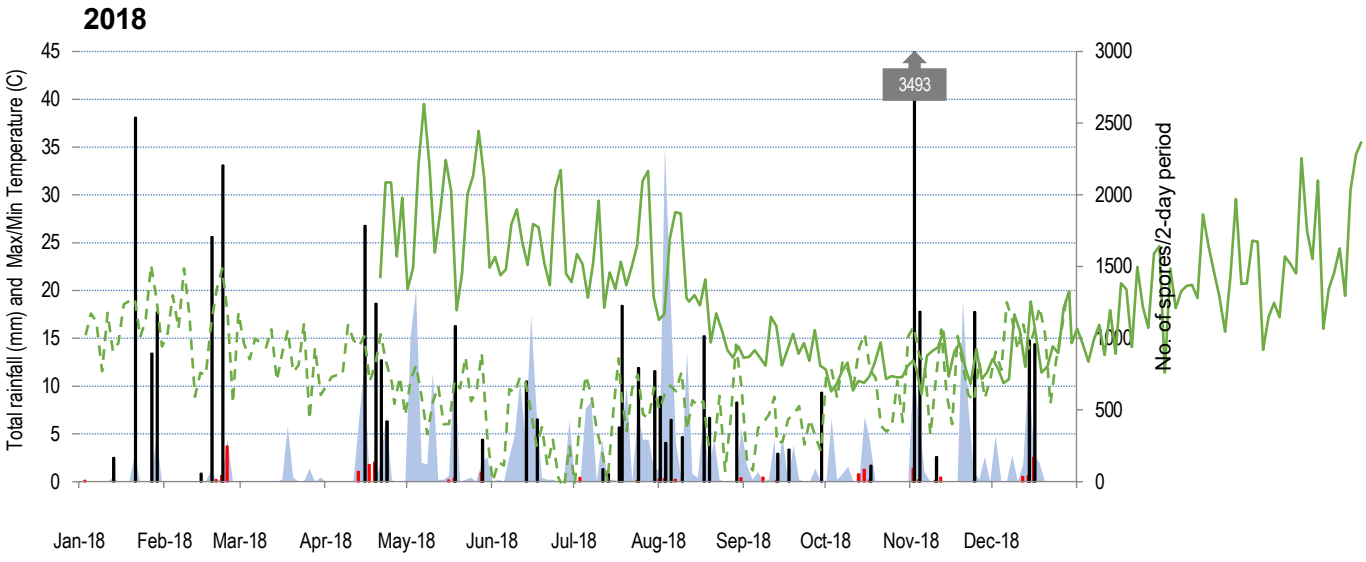
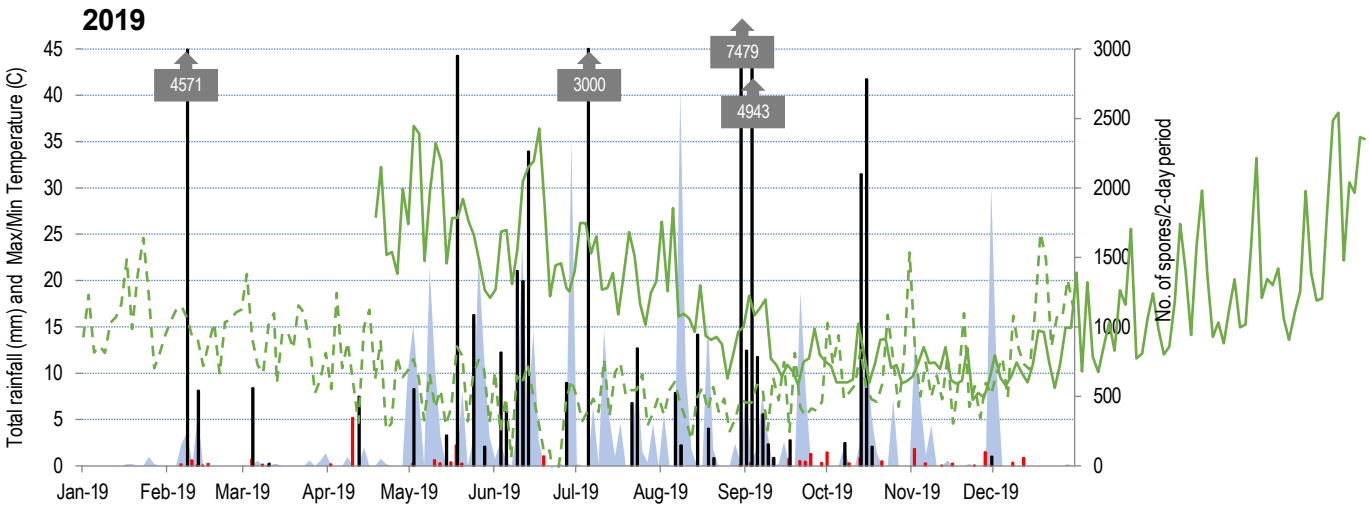


Figure 6.9. Number Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in Coonawarra, SA, from January 2017 to December 2021 and plotted against rainfall and maximum temperature (www.bom.gov.au).


2017
Rain
BD
ED
Min Temp
Max Temp
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
Spore trap deployed
Spore trap malfunction
3492
7989
3798
5205
3621
5313
Jan-17
Feb-17
Mar-17
Apr-17
May-17
Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17


2018
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
3493
Jan-18
Feb-18
Mar-18
Apr-18
May-18
Jun-18
Jul-18
Aug-18
Sep-18
Oct-18
Nov-18
Dec-18


2019
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
4571
3000
7479
4943
Jan-19
Feb-19
Mar-19
Apr-19
May-19
Jun-19
Jul-19
Aug-19
Sep-19
Oct-19
Nov-19
Dec-19

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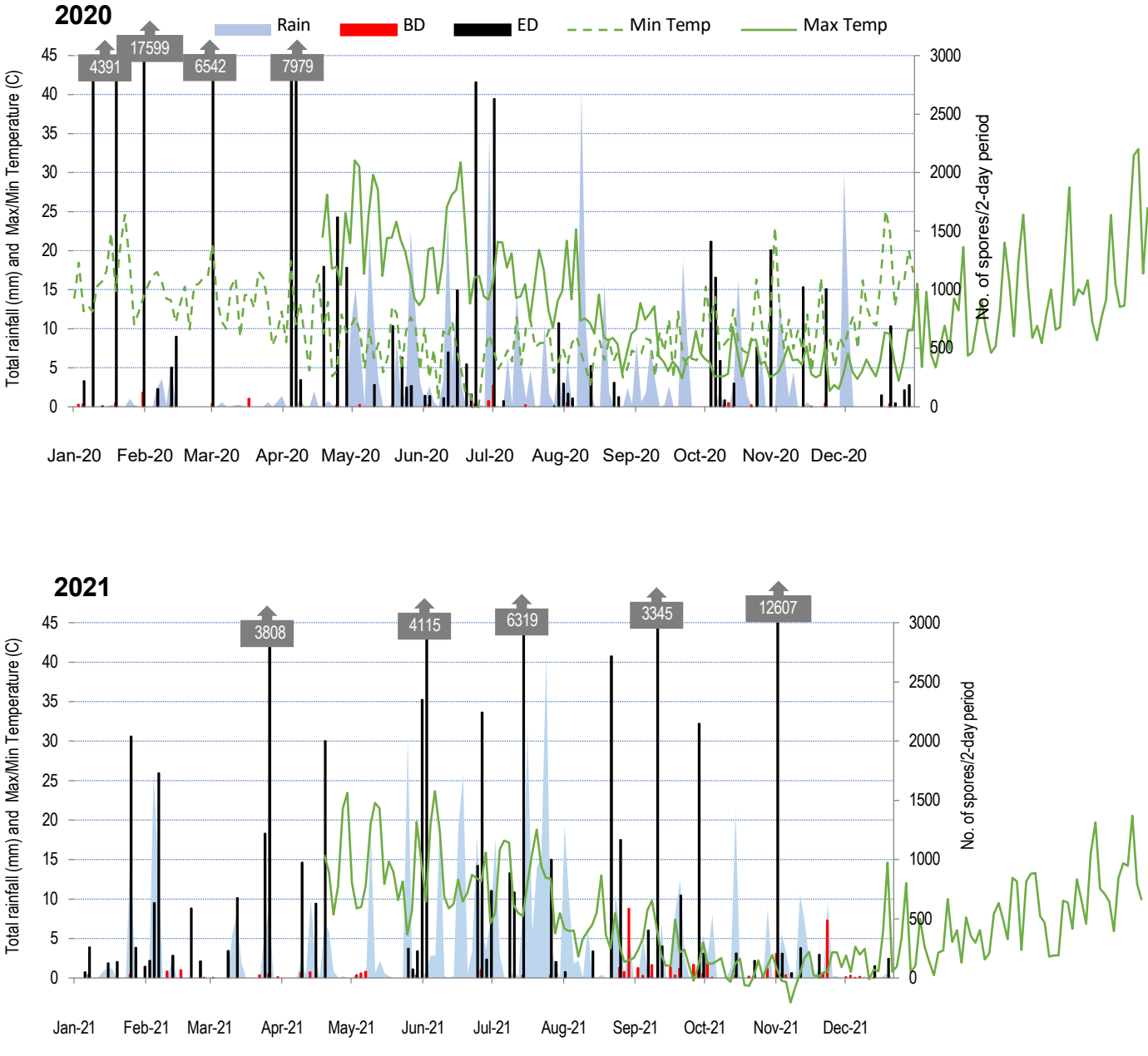
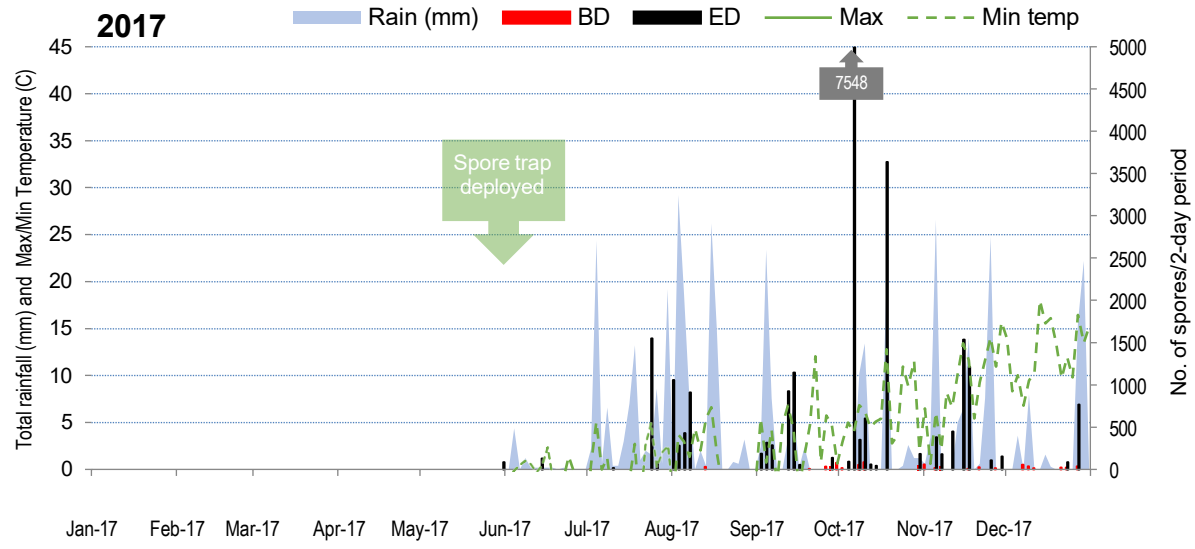
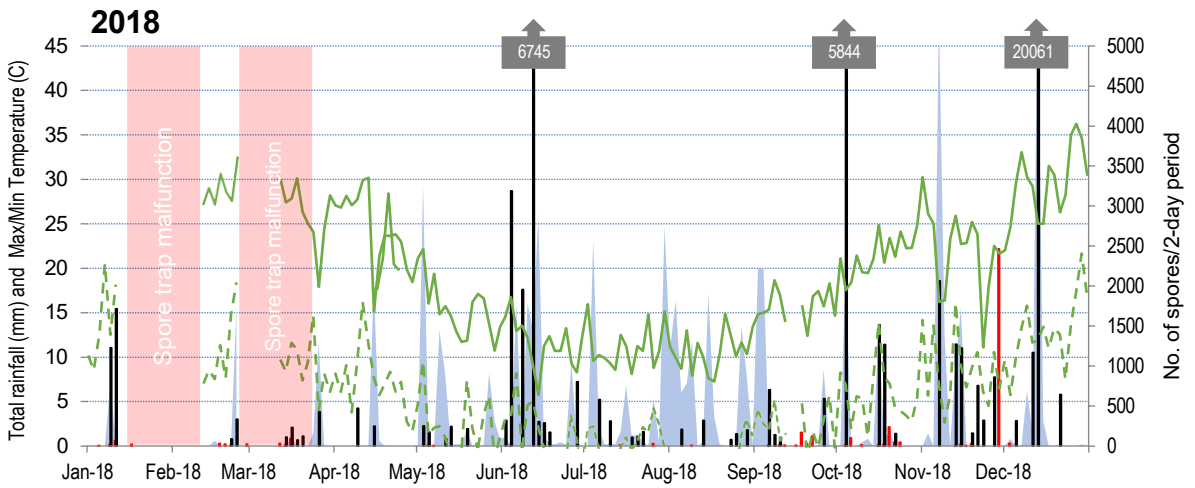
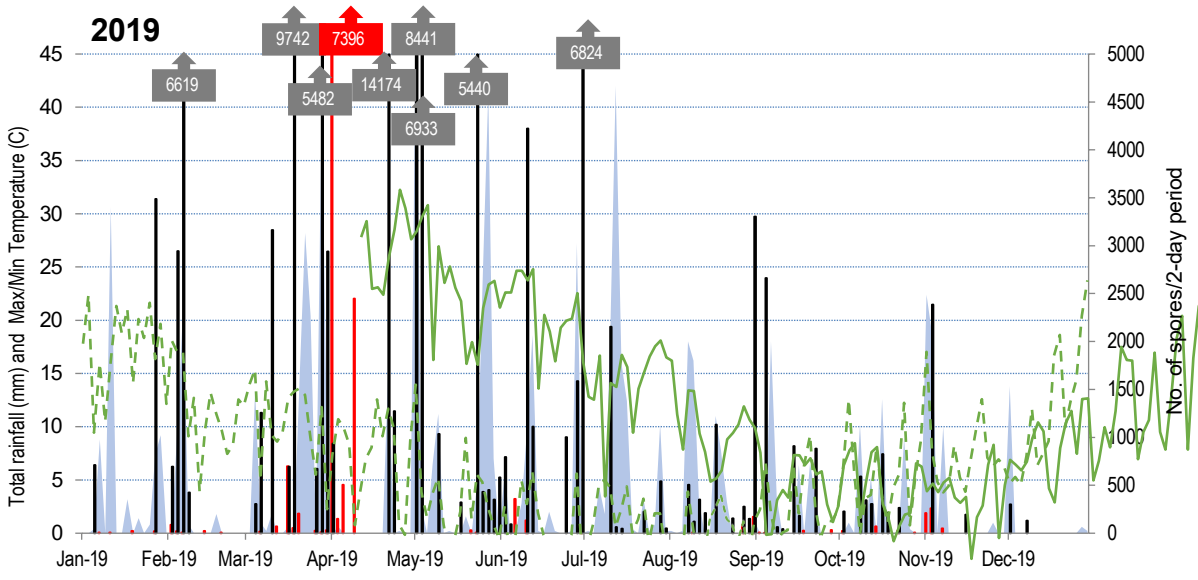


Figure 6.10. Number of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in McLaren Vale, SA, from May 2017 to December 2021 and plotted against rainfall and maximum temperature (McLaren Vale Grape Wine and Tourism Association).


2017
Rain (mm)
BD
ED
Max
Min temp
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
Spore trap deployed
7548
Jan-17
Feb-17
Mar-17
Apr-17
May-17
Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17


2018
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
Spore trap malfunction
Spore trap malfunction
6745
5844
20061
Jan-18
Feb-18
Mar-18
Apr-18
May-18
Jun-18
Jul-18
Aug-18
Sep-18
Oct-18
Nov-18
Dec-18


2019
Total rainfall (mm) and Max/Min Temperature (C)
No. of spores/2-day period
6619
9742
7396
8441
5482
14174
5440
6933
6824
Jan-19
Feb-19
Mar-19
Apr-19
May-19
Jun-19
Jul-19
Aug-19
Sep-19
Oct-19
Nov-19
Dec-19

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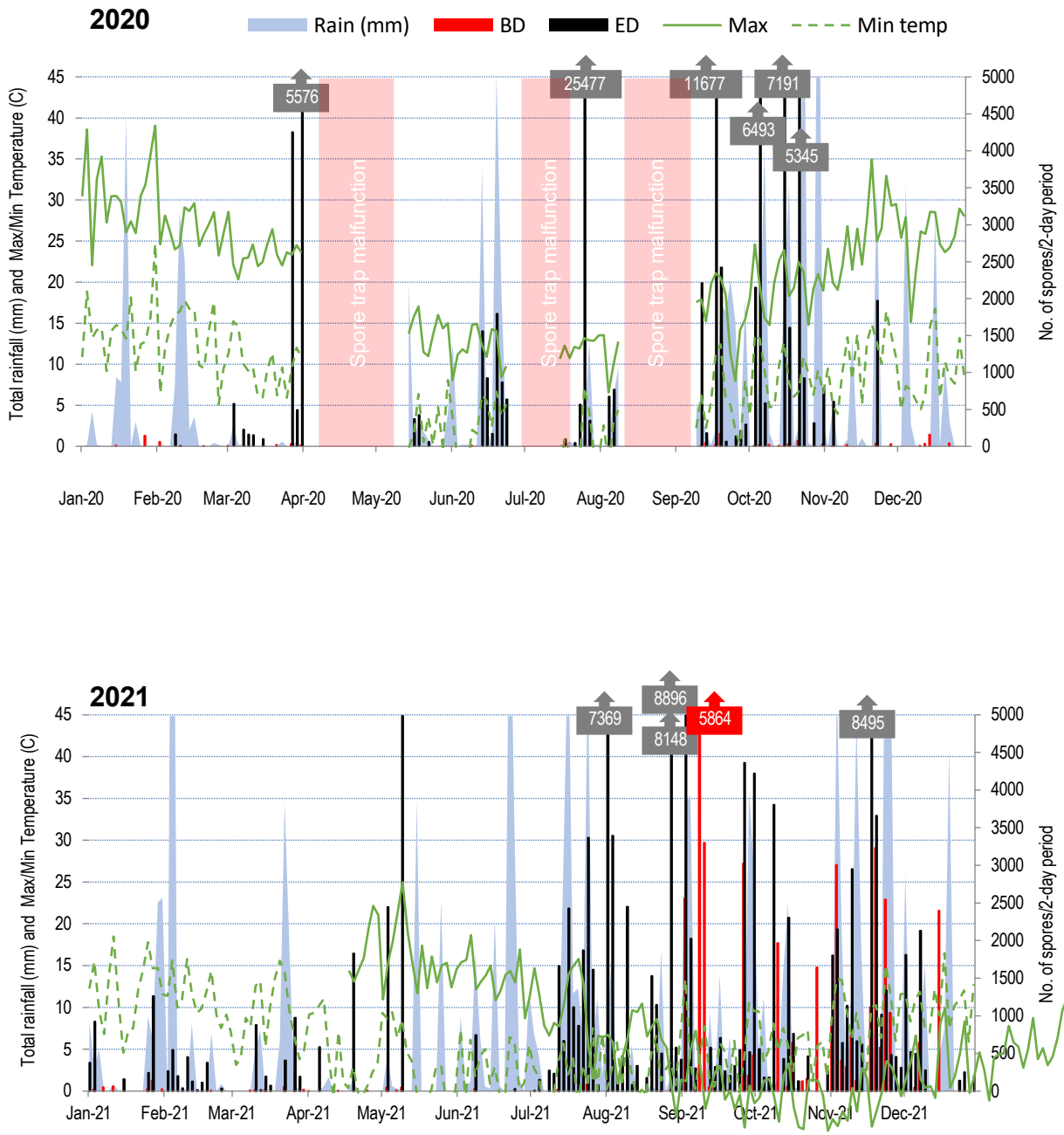


Figure 6.11. Number Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogen spores detected per two-day period in Tumbarumba, NSW, from June 2017 to December 2021 and plotted against rainfall and maximum temperature (www.dpi.nsw.gov.au/agriculture/horticulture/grapes/weather-stations-network/wsn).

6.1.3.2 Climatic factors that trigger spore release

2013-2016 (SAR 1205)

At all four sites, ED and BD pathogen spores were generally trapped during or immediately after rainfall (Figure 6.12 and Figure 6.13). As little as 0.2 mm of rain resulted in spore release (data not shown). Occasionally, ED and BD pathogen spores were continuously trapped for up to 14 days, which also coincided with continuous rain. However, not all rain events resulted in spore release of either pathogen, with several periods where no spores were trapped despite continuous rain occurring (Figure 6.12 and Figure 6.13). The number of two-day periods when BD pathogen spores (Figure 6.13) were not detected when rain was recorded was generally higher compared to those with ED pathogen spores (Figure 6.12) and was particularly evident in the Barossa Valley. Sometimes, ED and BD pathogen spores were detected in the absence of rain (i.e. at least four days before or after a rain event), particularly in Griffith and the Hunter Valley, NSW (Figure 6.12 and Figure 6.13).

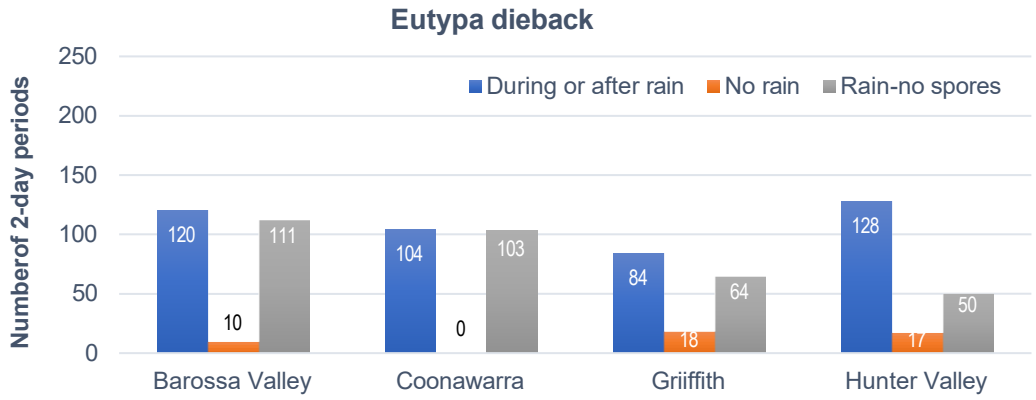


Figure 6.12. Total number of two-day periods between 2013 and 2016 with Eutypa dieback pathogen spores detected; during or within 48 hours of a rain event, in the absence of rain (i.e. at least four days before or after a rain event) and when no spores were detected and rain was recorded, from four wine growing regions in Australia.

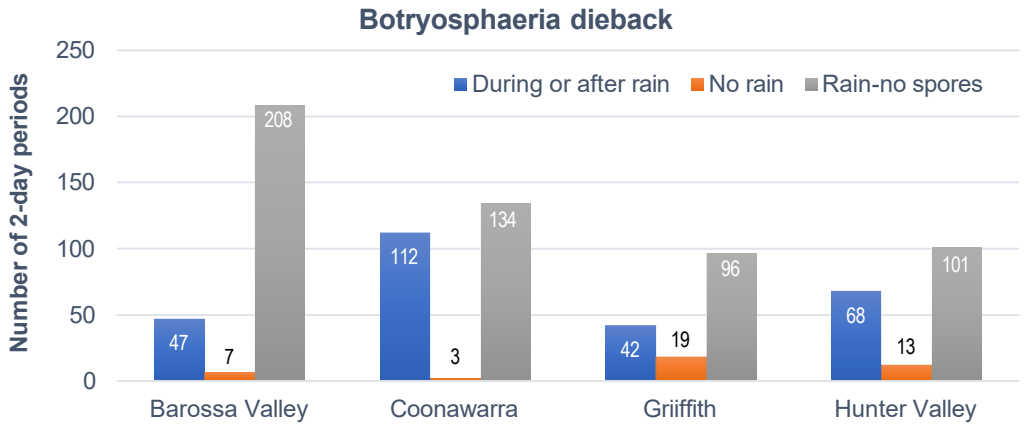


Figure 6.13. Total number of two-day periods between 2013 and 2016 with Botryosphaeria dieback pathogen spores detected; during or within 48 hours a rain event, in the absence of rain (i.e. at least four days before or after a rain event) and when no spores were detected and rain was recorded, from four wine growing regions in Australia.

Preliminary computer modelling of spore trap data from 2013-2016 (SAR 1205) was attempted by Simon McDonald (Spatial Analysis Unit, Charles Sturt University) to further investigate the effects of different climatic factors and the number of ED and BD pathogen spores trapped in each period. Spore counts for ED and BD pathogens were plotted against climatic factors (maximum and minimum temperatures, rain, humidity, dew point and wet bulb temperatures, maximum and average wind speed, vapour pressure) for all four sites. Preliminary modelling of the Hunter Valley data showed very weak or no obvious correlations between ED and BD spore numbers in any of the climatic factors analysed, including rain (Figure 6.14 and Figure 6.15). For instance, in the Hunter Valley, a 2.8 mm rain event in March 2016 resulted in the greatest number of ED pathogen spores (~30,000), while 57 mm of rain in January 2016 resulted in only 500 spores during that period.

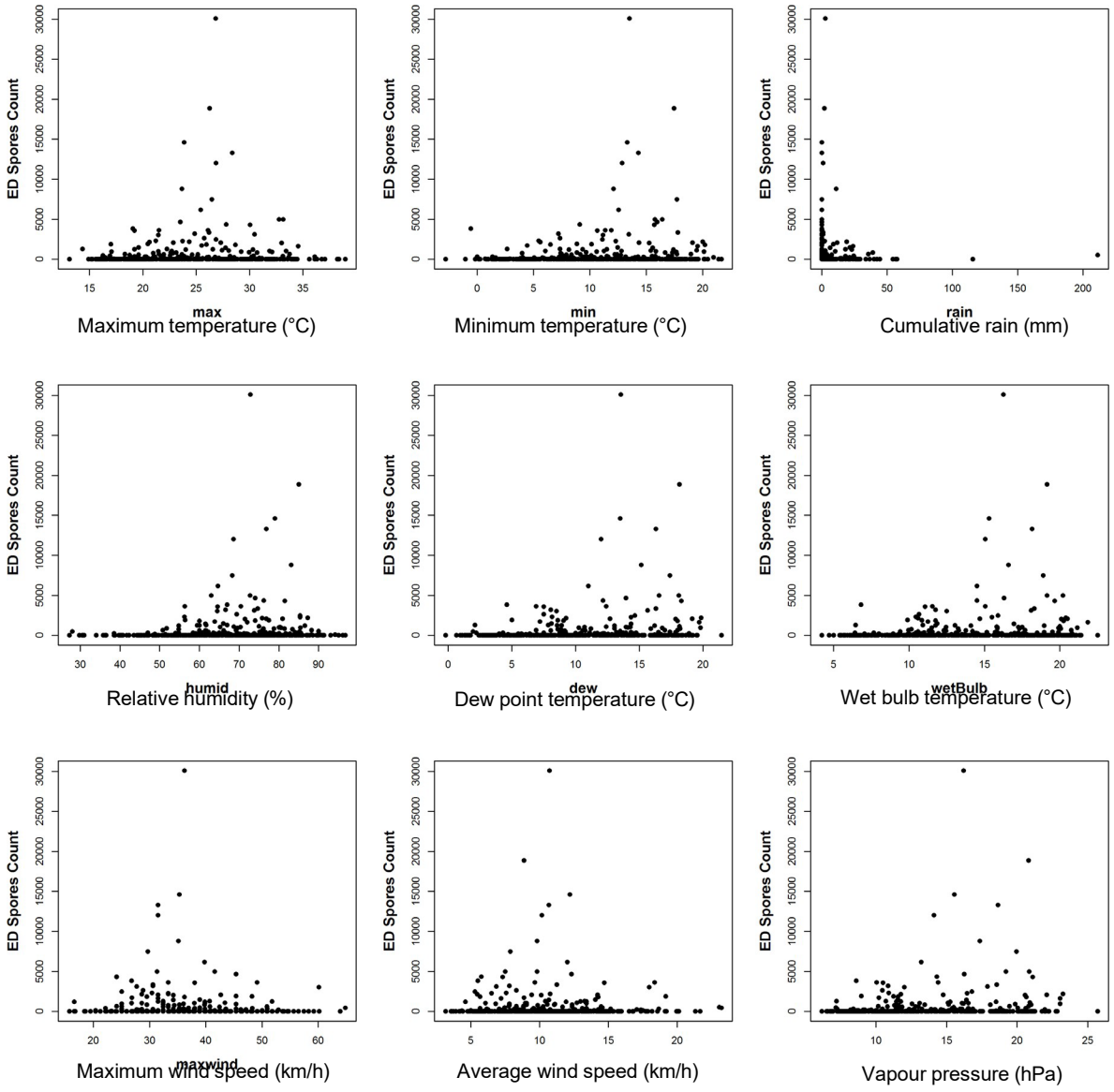


Figure 6.14. Eutypa dieback pathogen spore numbers detected from the Hunter Valley, NSW between 2014 and 2016 showing weak correlations when plotted against different climatic factors recorded for each two-day period (www.bom.gov.au).

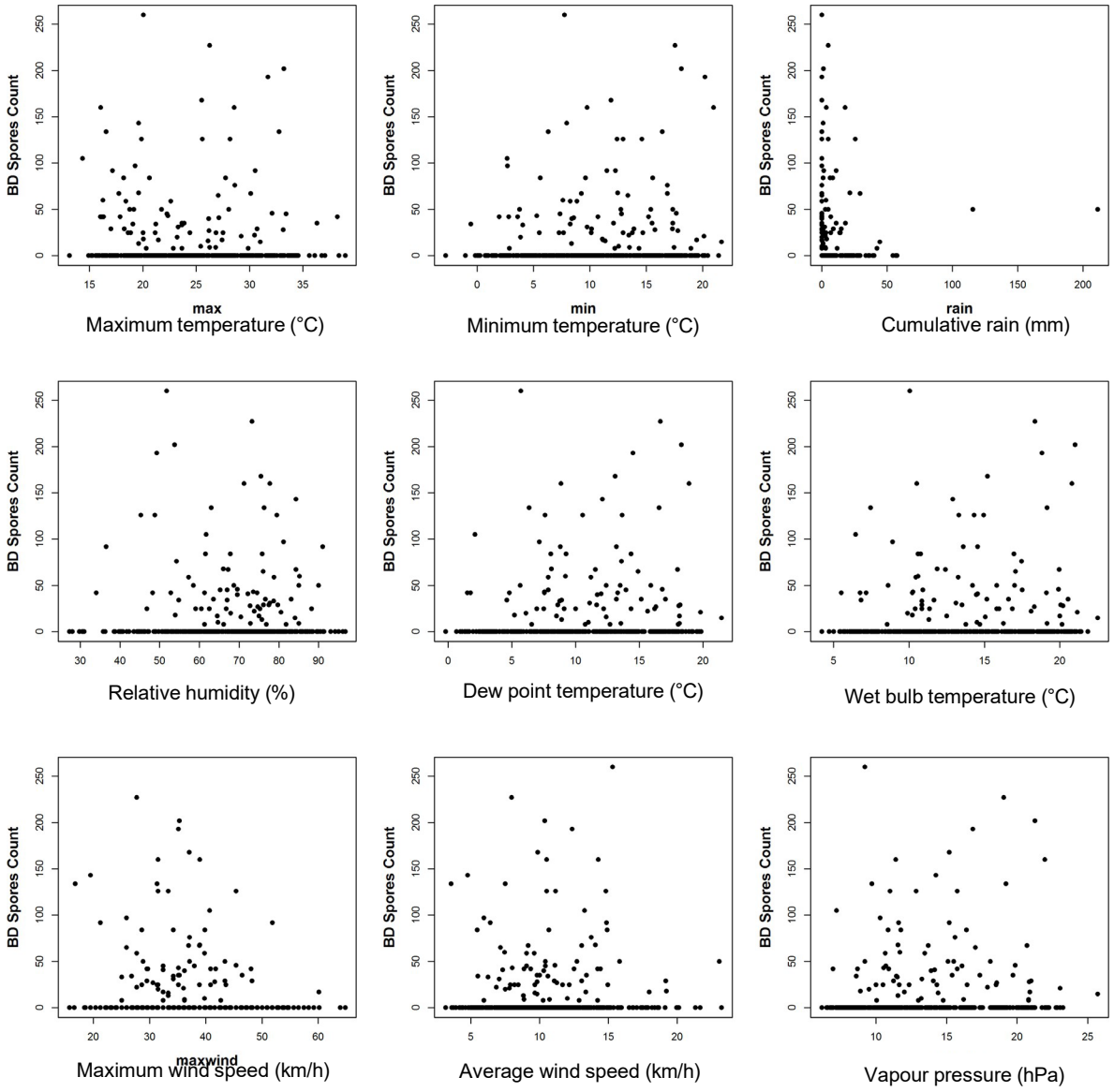


Figure 6.15. Botryosphaeria dieback pathogen spore numbers detected from the Hunter Valley, NSW between 2014 and 2016 showing very weak correlations when plotted against different climatic factors recorded for each two-day period (www.bom.gov.au).

2017-2019

Data from 2017-2021 confirmed the results from 2013-2016 (SAR 1205), attributing rain as the primary factor for the release of ED and BD pathogen spores. At all six sites, ED and BD pathogen spores were generally trapped during or immediately after the occurrence of rain (Figure 6.16 and Figure 6.17). The data further showed that not all rain events resulted in spore release particularly for BD pathogen spores, with several two-day periods where no spores were trapped despite rain being recorded (Figure 6.17). The number of two-day periods when BD pathogen spores (Figure 6.17) were not detected when rain was recorded was generally higher compared to those with ED pathogen spores (Figure 6.16), particularly evident in the Adelaide Hills and Coonawarra. On several occasions, ED and BD pathogen spores were trapped in the absence of rain (i.e. at least four days before or after a rain event) for all six sites (Figure 6.16 and Figure 6.17). Similar to the SAR 1205 results, there were no obvious correlations between different climatic factors and spore release of either pathogen group.

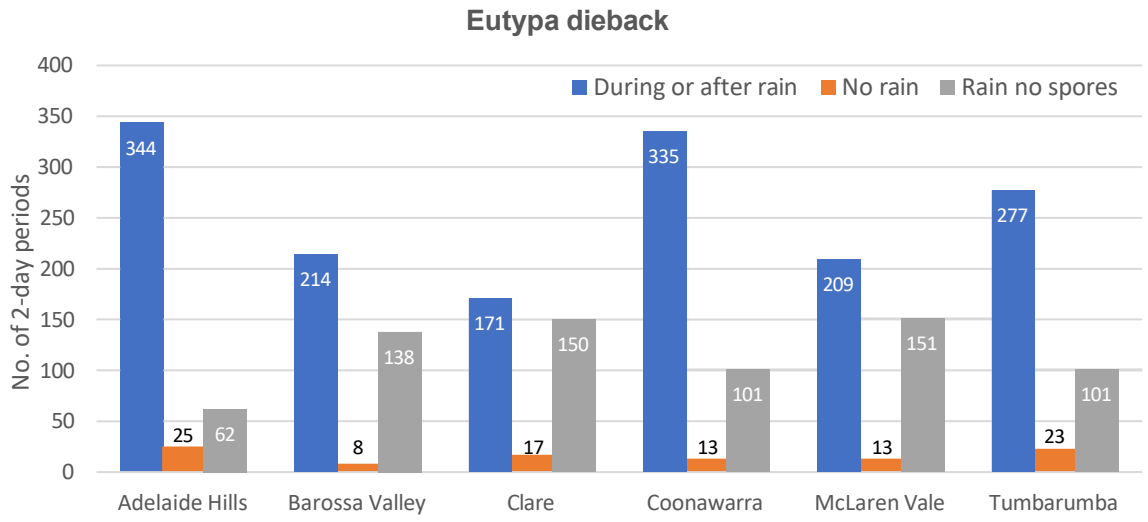


Figure 6.16. Total number of two-day periods between 2017 and 2021 with Eutypa dieback pathogen spores detected; during or within 48 hours of a rain event, in the absence of rain (i.e. at least four days before or after a rain event), and when no spores were detected and rain was recorded, from six wine growing regions in Australia.

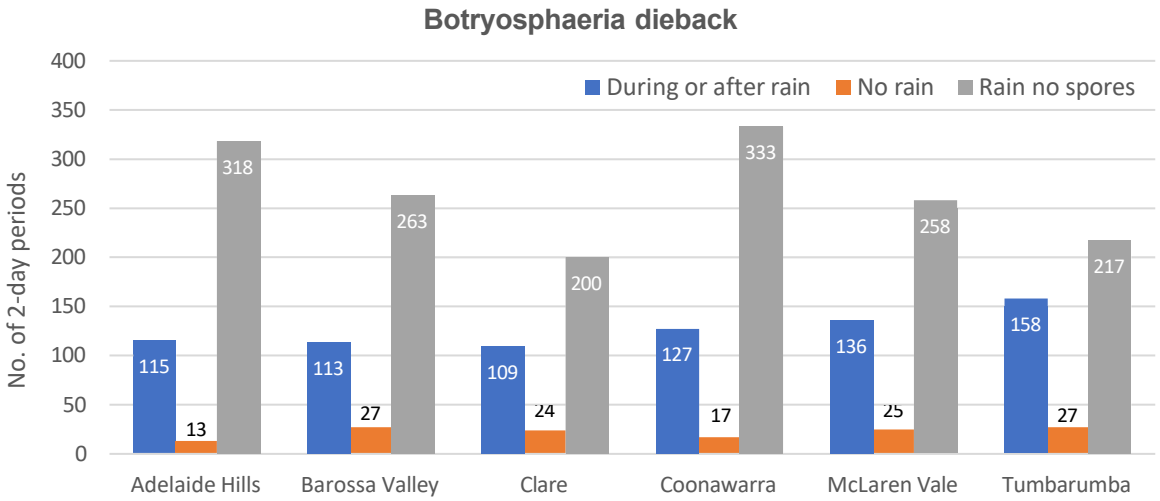


Figure 6.17. Total number of two-day periods between 2017 and 2021 with Botryosphaeria dieback pathogen spores detected; during or within 48 hours of a rain event, in the absence of rain (i.e. at least four days before or after a rain event), and when no spores were detected and rain was recorded, from six wine growing regions in Australia.

6.1.3.3 Identification of Diatrypaceous spores from vineyards

To investigate the diversity of ED pathogen spores detected in different regions, representative samples that tested positive using the ED pathogen multi-species primers in conventional PCR, were sent for DNA sequencing. Of the 217 PCR products sent for DNA sequencing, 70 (32%) resulted in poor or mixed sequencing reads which may be due to mixed DNA amplified by multi-target primers DITS1F and DITS 1R, and thus, were considered unknown. The remaining 147 samples yielded clean 250-300 bp DNA sequences allowing identification of each species. The BLAST analyses of the sequences identified seven Diatrypaceous species, but prevalence and distribution varied between regions (Figure 6.18). Four to five species were detected in all regions except for Coonawarra and Griffith where only two species were detected. *Eutypa lata* was the most prevalent species detected in seven out of eight regions, with highest incidence in the Barossa Valley (n=15), McLaren Vale (n=10), Adelaide Hills (n=9) and Coonawarra (n=8). One sample from Hunter Valley, NSW was detected with *E. lata*, despite *E. lata* not being reported in the region in previous studies (Creaser *et al*. 2003; Trouillas *et al*. 2011). *Diatrype stigma* was also prevalent, being detected in seven regions with highest incidence in the Coonawarra (n=13) and the Adelaide Hills (n=7). *Cryptovalsa ampelina* and *Eu. microtheca* were detected in five out eight regions with highest incidence in Clare (n=9) and Griffith, NSW (N=12) for *C. ampelina* and *Eu. microtheca*, respectively. *Cryptosphaeria multicontinentalis* was detected in three regions with highest detection in Tumbarumba (n=4). *Eutypella citricola* was only detected in two regions with highest incidence in the Hunter Valley, NSW (n=8). This is the first report of *Diatrype stigma* and *Cryptosphaeria multicontinentalis* in Australian vineyards.

Due to limited time and resources, identification of BD airborne spores by Sanger sequencing was not possible but will be incorporated in future spore surveillance research.

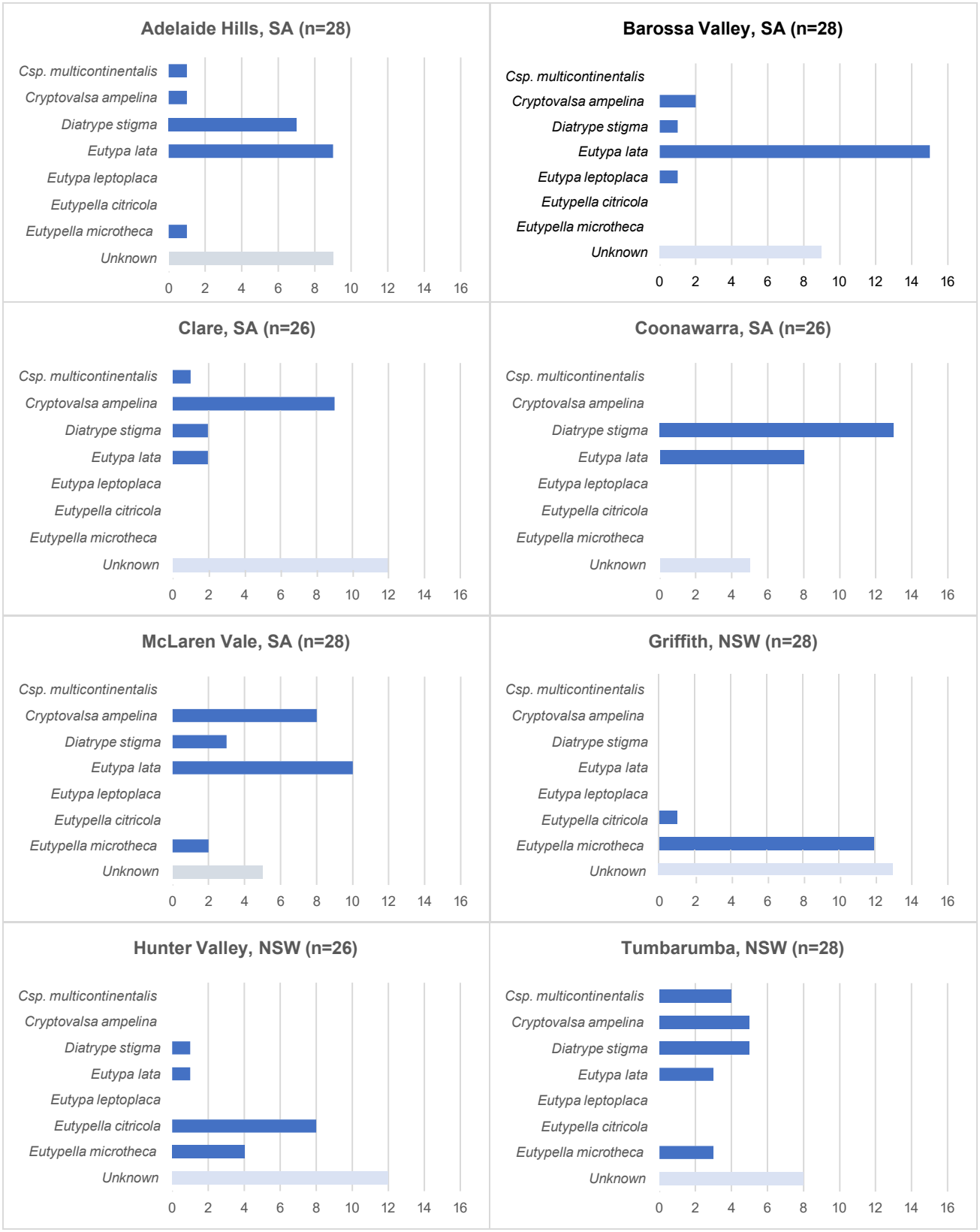


Figure 6.18. Species diversity of Diatrypaceous airborne spores from different wine growing regions in Australia. Airborne spores were collected using a Burkard volumetric spore trap and DNA extracted from two-day spore tape sections were identified by Sanger sequencing using Diatrypaceous multi-target primers DITS1F and DITS1R that can amplify 300-350 bp of the ribosomal DNA ITS region. Unknowns are the DNA samples with poor or mixed sequencing reads. *Csp.* is the abbreviation for the genus *Cryptosphaeria*.

6.1.4 **Discussion**

This study was the first attempt to comprehensively investigate the spore release patterns of both ED and BD pathogens, with more than 7000 spore trap DNA samples collected from eight Australian wine growing regions over eight years using multi-faceted molecular tools. The spore trapping study in 2013-2016 (SAR 1205) showed spore dispersal of ED and BD pathogens was sporadic and varied between regions, seasons and years. In most cases, spores were primarily detected in late autumn to early spring, while spores were also trapped over summer on a few occasions. Spores were generally detected during or within 48 hours of rain but not all rain events resulted in spore detection. The spore numbers and frequency of detection varied between years, with the highest number of spores being recorded in 2016, particularly for ED pathogens. The subsequent spore trapping studies in six regions from 2017 to 2021 in the current project continued to show that spore dispersal of ED and BD pathogens was sporadic, which also varied between regions, seasons and year. The spore numbers and frequency of detection also varied between years, with the highest number of spores being recorded in 2021 in the Adelaide Hills, Clare, Coonawarra and Tumbarumba, particularly for ED pathogens.

Studies from overseas revealed that the seasonal release of these spores varied depending on the climate of each region (Amponsah *et al.* 2009; Kuntzmann *et al.* 2009; Urbez-Torres *et al.* 2010; van Niekerk *et al.* 2010), which was similar to the results obtained from the different wine regions in Australia. Spore trapping using PVC guttering during the month of August from 2009 to 2011 in the Hunter Valley also detected viable BD spores, indicating that the release of BD spores was highly correlated with rain (Wine Australia 2013). Overall, the results from the two spore trapping studies showed the number of two-day samples with detection of ED pathogens was higher than that of BD pathogens in most regions, except for Clare where the number of samples detected with BD pathogens was higher. However, the number of samples with pathogen detection also varied between years. For instance, the number of samples detected with BD pathogens in Coonawarra was higher in 2013-2016 (23%) but decreased in 2017-2021 (17%). In Tumbarumba, the number of samples detected with BD pathogens was also higher in 2021 (31%) compared to other seasons (21-23%). ED pathogen detection was extremely high in the Hunter Valley in early 2016 while BD pathogens were rarely detected during this period. Furthermore, ED detection in the Adelaide Hills, Clare and Coonawarra were extremely high in 2021 (54-59%), while BD pathogen detection was relatively low (11-24%). The high detection of ED pathogens in the Hunter Valley was unexpected since *E. lata* is believed to be more prevalent in cooler regions (Highet and Wicks, 1998). However, this species has not been reported from surveys in the Hunter Valley (Creaser *et al*. 2003; Trouillas *et al.* 2011), which has a sub-tropical climate. The high number of ED pathogen spores trapped in the Hunter Valley may be attributed to other Diatrypaceae species, since the representative samples from Hunter Valley were predominantly identified as *Eu. citricola* and *Eu. microtheca* using DNA sequencing and LAMP assays, and with only one spore tape sample detected with *E. lata*. Trouillas *et al.* (2011) reported that *Eu. citricola and Eu. microtheca* were the most prevalent species identified in the Hunter Valley while *E. lata* was not isolated from any grapevines in this region.

At all spore trapping sites, ED and BD pathogen spores were generally detected during or immediately after the occurrence of rain, with as little as 0.2 mm of rain resulting in spore release. Moller and Carter (1965) showed that *E. lata* ascospores were released following as little as 1.2 mm of rain. Occasionally, ED and BD pathogen spores were continuously detected for up to 14 days, which also coincided with continuous rain. However, not all rain events resulted in spore release of either pathogen, with no spores being detected over several periods despite continuous rain occurring. The main outcome from this study showed rainfall as a primary factor that triggers the release of either ED or BD spores, which is in general agreement with past studies in vineyards in California (Petzoldt *et al*. 1983; Ramos *et al*. 1975; Trouillas 2009; Urbez-Torres, *et al*. 2010), Michigan (Trese *et al.* 1980) and New York (Pearson 1980) in the United States, as well as France (Kuntzmann *et al*. 2009) and South Africa (van Niekerk *et al.* 2010). On some occasions, however, spores were also trapped several days after a rain event, particularly for BD pathogen spores. The detection of spores in the absence of rain suggests other climatic factors such as relative humidity or dew point may also play a role in the release of these spores.

The studies previously described from overseas also revealed the seasonal release of ED and BD pathogen spores to vary depending on the region. In California, the greatest number of BD pathogen spores were trapped primarily in autumn and winter, however spore numbers declined significantly in late winter and early spring (Urbez-Torres *et al*. 2010). These seasonal differences in spore release

prompted recommendations to delay pruning until later in the season to reduce the risk of pruning wounds becoming infected by these spores (Urbez-Torres *et al*. 2010). However, very few or no BD and ED pathogen spores were trapped in France (Kuntzmann *et al*. 2009) and Michigan (Trese *et al*. 1980), respectively, during winter because of sub-freezing weather conditions. Therefore, delaying pruning in these regions may not make any difference in minimising the risk of infections. Furthermore, BD pathogen spore release was shown to peak during vegetative growth of the vine (Kuntzmann *et al*. 2009; Amponsah, *et al*. 2009), thus, any wounds created as part of canopy management (i.e. sucker removal, shoot trimming) may also be at risk of becoming infected by these spores. It is important to note that the previous spore dispersal studies described above relied primarily on conventional techniques such as microscopy and culturing of the microorganisms on artificial media, which are limited in accuracy and sensitivity in detecting these pathogens from environmental samples (Kuntzmann *et al.* 2009; Úrbez-Torres *et al.* 2010; van Niekerk *et al.* 2010), and thus may have affected spore detection in certain periods.

Despite the findings from the current spore trapping studies, and studies conducted overseas indicating rain as a primary factor in the release of ED and BD pathogen spores, preliminary results from the computer modelling showed very weak correlation between the incidence of spore release and numbers of spores and the occurrence of rain. This weak correlation is most likely due to the high number of rain events where spores were not detected, particularly for BD. High spore numbers were also detected regardless of the amount of rain recorded. Furthermore, on a few occasions at different locations, spores were trapped more than a week after any rainfall event. Other weather factors such as temperature, relative humidity, dew point and wind speed also showed weak correlation with spore detection, indicating that spore release may be difficult to predict for these pathogens. In recent years, data-mining techniques using decision tree algorithm software have become a powerful tool in developing prediction models using a large amount of data (Rahman, *et al.* 2019). Consultation with a data-mining specialist may further assist in identifying climatic factors that contribute to the release of ED and BD spores and further elucidate the spore release patterns of these pathogens.

The lack of obvious correlations between climatic factors and spore release may also be due to the amount of time required for the pathogens to develop new fruiting structures. The fruiting bodies of the ED and BD pathogens are generally known to survive in infected wood for a long period of time and produce spores when environmental conditions are favourable for their release. For example, the stroma of *E. lata* can survive for more than one year (Moller and Carter 1965) and can produce new spores approximately 12 days after being exhausted (Dubos 1987; cited by Carter 1991). Botryosphaeriaceae species were also shown to survive in dried wood pieces after three months in storage at room temperature and produced new pycnidia and conidia when exposed to 36 hours of high humidity (Amponsah *et al*. 2008). However, further investigation is required to determine the duration of the production and maturation of perithecia and/or pycnidia of these pathogens. These future studies may further elucidate the biology of these pathogens and the effect of environmental factors on their development and spread.

Another important observation obtained from this study was the difference in the number of spores released by ED and BD pathogens, with larger numbers of ED spores detected (up to 30,000 spores) in a two-day sample compared to that of BD spores. This difference in spore numbers may be due to the size and mode of discharge of these spores. It is well known that *E. lata* and its closely related species can actively eject their ascospores from their fruiting structures into the air and these can travel long distances (potentially up to 160 km) depending on wind speed (Moller and Carter 1965). In contrast, the spores of BD pathogens are released in slimy ribbon-like spore masses (Slippers and Wingfield, 2007) and the impact of rain droplets is necessary for spores to be dispersed (Urbez-Torres *et al*. 2010). Since the BD spores are less airborne, the distance of their dispersal in vineyards is most likely shorter than for ED spores (Urbez-Torres *et al*. 2010). Baskarathevan *et al*. (2013) showed Botryosphaeriaceae spores can be rain-splashed up to two metres away from the inoculum source, however this study did not assess airborne inoculum. The current study detected BD pathogen spores in a trap located 20 m from the closest inoculum source, which, to our knowledge, is the first report of BD pathogen spores becoming airborne for these distances. In terms of spore sizes, the ascospores of *E. lata* and other Diatrypaceae species (8-12 × 2-3 µm; Trouillas *et al*. 2011) are less than half the size of BD spores (22-28 × 6-12 µm; Pitt *et al*. 2010), thus making Diatrypaceae spores smaller and lighter for wind dispersal. Despite the difference in spore sizes, the diameter of fruiting structures of the ED pathogens (250-450 µm; Trouillas *et al*. 2011) is similar to that of BD pathogens (200-500 µm; Phillips, *et al*. 2013). It is therefore possible that the fruiting structures of ED pathogens can produce and contain more ascospores, thus more spores are released, than that of the BD pathogens. Regardless, even if spore numbers are low, both ED and BD spores can still infect susceptible wounds. Moller and Carter (1965)

showed that as few as 10 *E. lata* ascospores can result in infection in apricots. Elena *et al*. (2015) reported that inoculation with 10 spores of *E. lata* and *D. seriata*, resulted in 20 and 40% recovery from wounds, respectively. Amponsah *et al*. (2014) also showed that as few as two *N. luteum* conidia can result in infection of grapevine shoots.

DNA sequencing of spore tape samples testing positive to ED spores identified seven species, with *E. lata* being present in seven out of eight regions, and also the most prevalent species in three SA regions. However, *Cryptovalsa ampelina* and a newly reported species, *Diatrype stigma*, outnumbered *E. lata* in two other SA regions. *Eutypella microtheca* and *Eu. citricola* were most prevalent in two New South Wales (NSW) regions while being rarely detected in SA. This study represents the first report of *D. stigma* and *Csp. multicontinentalis* in Australian vineyards. One isolate each of *Diatrype* and *Cryptosphaeria* has previously been isolated from grapevines in Tumbarumba, NSW and poplar in Khancoban, NSW, respectively, but were not identified to species level (Trouillas *et al.* 2011). It is likely that these species have been present in Australia without prior identification. *Diatrype stigma* has been reported as a pathogen of grapevine in California (Trouillas and Gubler 2010) while *Csy. multicontenitalis* has been reported as a pathogen of poplars in South Africa (Moyo *et al.* 2017). Since these two new species were only detected by PCR and not by fungal isolations from infected wood, their association with the dieback symptoms observed in vineyards warrants further investigation. Future research to identify different Botryosphaeriaceae species from spore tapes by DNA sequencing is also essential, to further elucidate their prevalence and diversity in different wine growing regions in Australia.

Pruning of wine grapes in Australia normally takes place during winter (June – August) when vines are dormant, which usually coincides with the highest rainfall periods. Pruning wound susceptibility studies showed that wounds were most susceptible to ED and BD pathogens during the first two weeks following pruning (Section 7.3.1; Sosnowski *et al*. 2017; SAR 1205). While wounds appear to be more susceptible to *E. lata* and *N. luteum* when pruned early, susceptibility to *D. seriata* did not differ regardless of pruning time. Since observed spore release patterns varied between regions and years, there may be little advantage in using different pruning times to minimise the risk of infections of these pathogens. However, pruning wound protection trials (Sosnowski *et al*. 2017; SAR 1205; Ayres *et al*. 2022) showed that fungicide treatments applied within one week of pruning can protect wounds from infection by *E. lata* and *N. luteum* for up to two weeks, providing a potential three-week window of protection. Since the wine regions in Australia cover a wide area with highly diverse climates, the comprehensive spore trapping in different wine regions in this study provided beneficial information on the spore release patterns of ED and BD pathogens in different regions. The data generated from samples analysed to date demonstrated that the seasonal spore release patterns of ED and BD pathogens varied between pathogens, regions and years. Rainfall was also identified as the primary environmental factor that influenced the release of these pathogen spores. Future spore trapping studies in other wine regions of Victoria, Tasmania and Western Australia will further elucidate the critical times of the year when ED and BD spores are abundant in vineyards in these regions. This will provide localised data for each region that will assist growers in making decisions for optimal pruning time and fungicide treatments of their vineyards.

6.2 Rotorod impaction spore traps

6.2.1 Introduction

Rotorod impaction spore traps, also referred to as rotating arm or whirling arm samplers, are used to monitor pollen or fungal spore dispersal over discrete time periods from hours to several days (Edmonds 1972, Aylor 1993, Lacey and West 2006, West and Kimber 2015). Rotorods have some advantages over Burkard volumetric spore traps. They are easy to deploy at different heights above the ground and they are inexpensive, so several can be deployed at a sampling site to compare treatments or spatial variation (Aylor 1993). Furthermore, they are not sensitive to changes in wind speed, making them ideal for measuring vertical spore gradients, as the wind speed typically increases with height above the ground. However, rotorods must be changed frequently to determine temporal patterns and it is difficult to accurately calculate the concentration of spores in the atmosphere (Edmonds 1972, Aylor 1993).

In this preliminary study, rotorod spore samplers were evaluated for use in vineyards to detect ED and BD pathogen spores.

6.2.2 Methods

6.2.2.1 Rotorod spore sampling

The rotorod spore trap (Burkard Manufacturing Co, Rickmansworth, England) consisted of a 12 Volt electric motor fitted to a rotating arm with two clips at either end for inserting clear plastic rods (Figure 6.20a). The leading edges of the plastic rods were smeared with Vaseline to capture spores, prior to being placed into the clips. A fan blade was located in the centre of the rotating arm to draw in air from beneath the aluminium dome that acts as a rain and wind shield to minimise spore impaction whilst the rotating arm was stationary (Figure 6.20b). The electric motor was connected to a controller that could be programmed to start and stop the motor, and the controller was connected to a 12V battery located in a waterproof plastic container on the ground (Figure 6.20c). The rotorod and controller were attached to a steel post at the desired height in the vineyard.

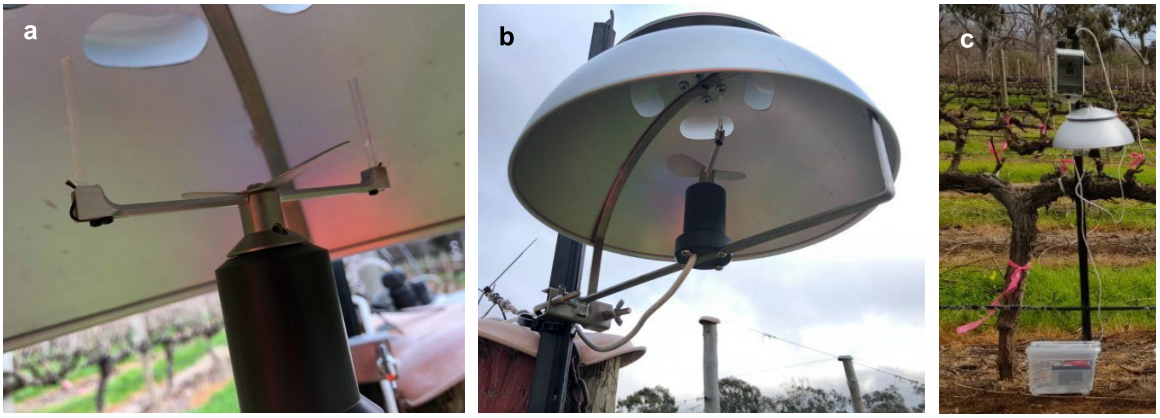


Figure 6.19. Rotorod spore trap (a-b) with rotating arm, two clear plastic rods and a fan blade to draw in air and (c) attached to a steel post with controller and 12 V battery in a plastic box in a vineyard.

6.2.2.2 Variation around a vineyard block

An experiment was established in a vineyard (cv. Shiraz, planted in 1993) located at the Waite Campus, Urrbrae, South Australia. Rotorod spore traps were set up in five locations in and around the vineyard block. Position 1 - 50 m south of the block, Position 2 - on the southern edge of the block, Position 3 - middle of the block, Position 4 – northern edge of block and Position 5 – eastern edge of block (Figure 6.21a). Position 3 consisted of a tall pole with four traps at heights of 40, 110, 190 and 250 cm from the ground (Figure 6.21b). At positions 1, 2 and 5, there were two traps at heights of 40 cm and 110 cm (Figure 6.21c-d), with the 40 cm height representing the height of the Burkard volumetric spore trap air intake orifice and 110 cm representing the pruning wound zone above the cordon. At position 4 there was only one trap at 110 cm, as no more traps were available.

On 13 August 2020, all spore traps were run for 3 h from 9 am to 12 pm (Run 1) and again from 12 pm to 3 pm (Run 2). Then traps were programmed to run from 12 am to 3 am on 14 August (Run 3). After each run, each rod was removed and stored in a labelled 1.5 mL plastic tube. Rainfall was predicted during the week of the experiment. Rainfall data were retrieved from an automatic weather station located 50 m from the vineyard block (www.greenbrain.net.au).

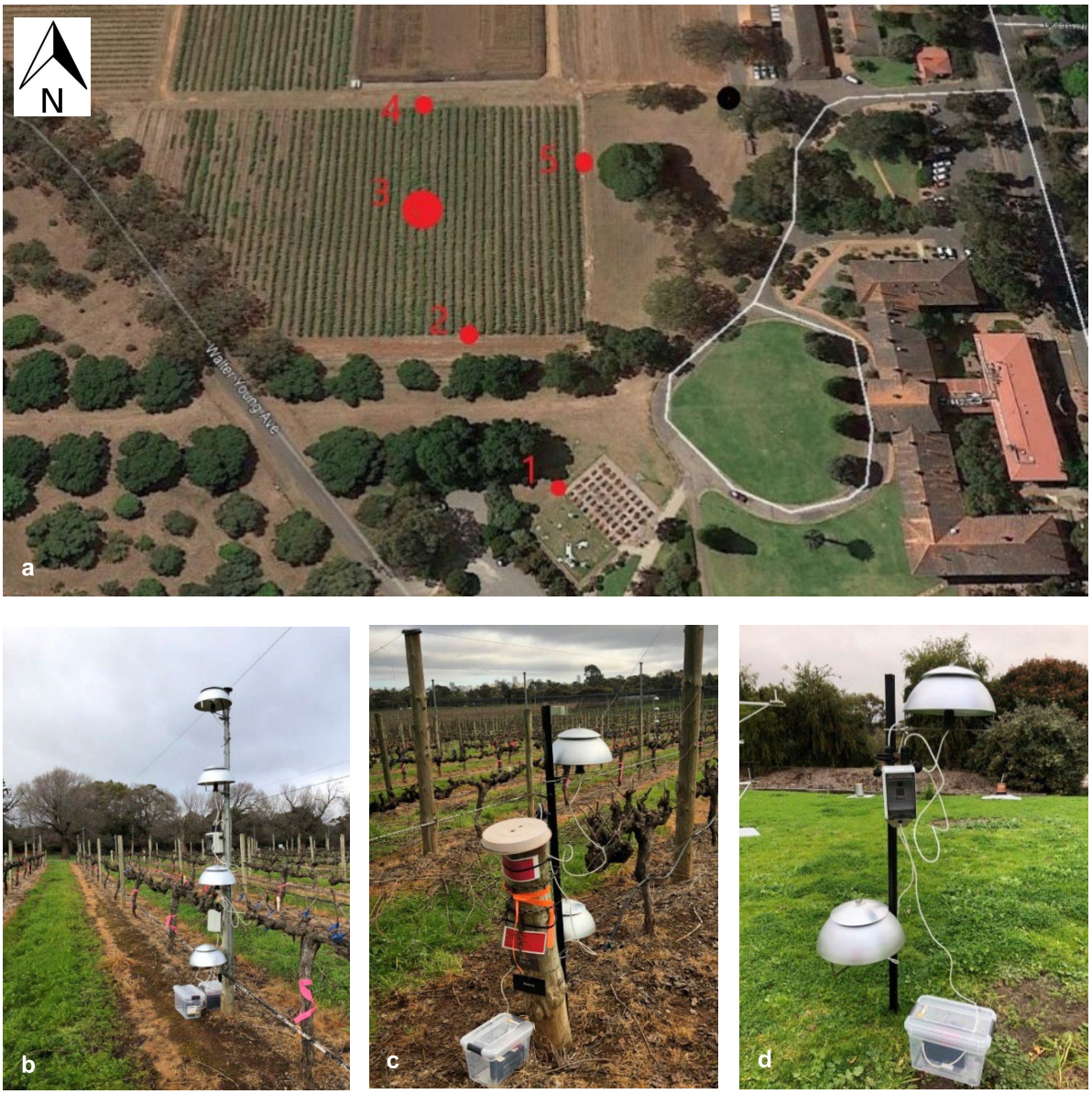


Figure 6.20. (a) Location of rotorod spore traps at five positions in and around a Shiraz vineyard block. (b) Position 3, four traps at various heights in the middle of the block. (c) Position 2, two traps at southern edge of block. (d) Position 1, two traps 50 m south of the block.

6.2.2.3 Sampling duration

A second experiment was established in the vineyard (Section 6.2.2.2). A series of six rotorods were attached to steel posts placed 50 cm apart with the base of domes approximately 1 m above the ground, directly above the cordon (Figure 6.22). Controllers were set to start and stop so that rotorods ran for durations of 1, 3, 6, 12, 18 or 23 h, with durations randomly assigned to posts. Beginning at 10 am on 17 August 2020, the first run commenced, and then on each of the following three days, runs were repeated. Between 9 and 10 am each day, each rod was removed and stored in a labelled 1.5 mL plastic tube, and a fresh set of rods was placed into traps. Batteries were also replaced with fully charged ones. Rainfall was predicted during the week of the experiment. Rainfall data were retrieved from an automatic weather station located 50 m from the vineyard block (www.greenbrain.net.au).



Figure 6.21. Six rotorod spore traps used to compare sampling times

6.2.2.4 Monitoring spores in a remediated vineyard

A third experiment was established in a vineyard block (Shiraz, planted in 2001) located near Greenock in the Barossa Valley, South Australia. In July 2020, 15 rows of the vineyard were subjected to remedial surgery by cutting trunks above the dripline at about 40 cm above the ground and completely removing all debris. A watershoot was selected in the following season to train onto the wire to become the new cordon. A further 13 rows to the east of the remediated vines were left untreated.

In August 2021, rotorod spore traps were deployed on steel posts in six positions throughout the vineyard, with three located within the remediated rows and three in the untreated vines (Figure 6.24a). Each post had two traps, at 50 and 110 cm heights, except at position 4 where there was only one trap at 110 cm. Posts with spore traps, controllers and batteries were placed midway between two vines (Figure 6.24b-c).

All traps were programmed to run for 24 h on six different days based on rainfall prediction with scheduled timing outlined in Figure 6.23. Spore rods were replaced before each run and stored in 1.5 mL plastic tubes.

Table 6.3. Program schedule for rotorod spore trap runs in a Shiraz vineyard near Greenock in the Barossa Valley, South Australia.

Run	Start date	Start time	End date	End time	Notes
1	11/8/21	9 am	12/8/21	9 am	
2	12/8/21	10 am	13/8/21	10 am	1, 2 not running on arrival, 3-5 running slowly
3	20/8/21	11 am	21/8/21	11 am	2 failed to operate correctly
4	21/8/21	12 pm	22/8/21	12 pm	
5	22/8/21	1 pm	23/8/21	1 pm	
6	23/8/21	2 pm	24/8/21	2 pm	2 failed to operate correctly

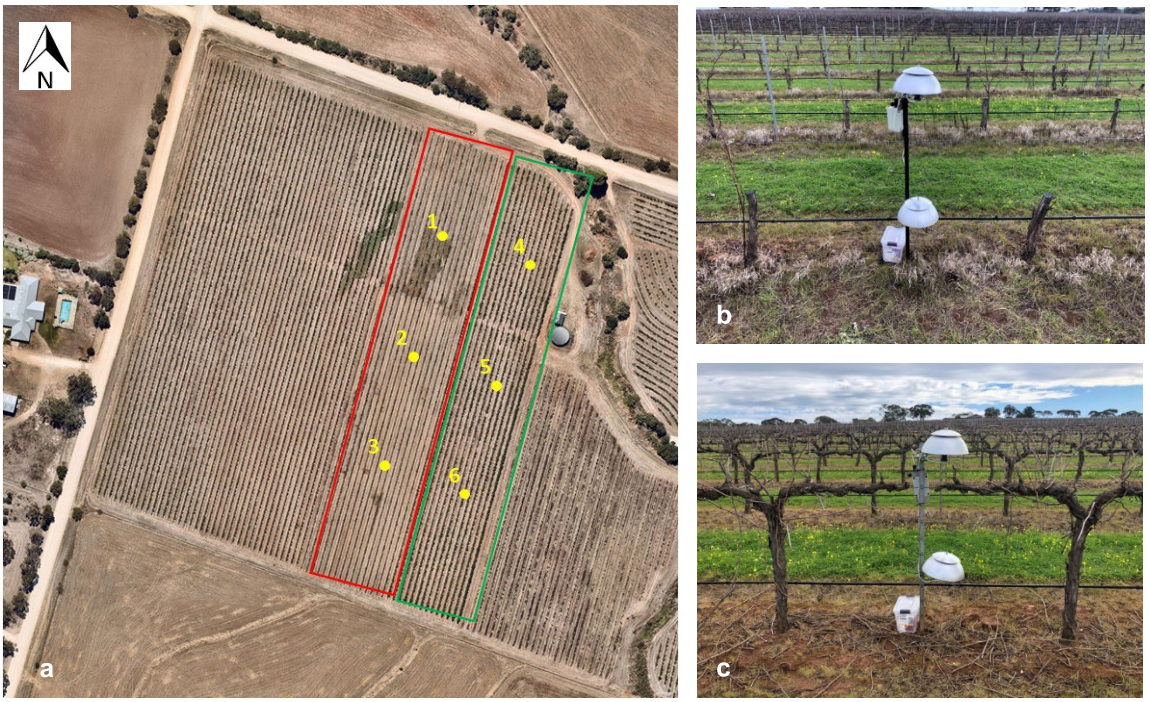


Figure 6.22. Vineyard with 15 rows having trunks cut to 40 cm outlined in red and 13 rows not subjected to remedial surgery outlined in green (a). Yellow dots and numbers represent positions of the rotorod spore traps. Each set of two spore traps was positioned between two vines that were remediated (b) or not (c).

6.2.2.5 Analysis of rods

Each rod was placed in a sterile 1.5 mL Eppendorf tube (lysing tube) containing ~300 mg of (400-600 µm diameter) Ballatoni beads (Daintree Scientific, Australia) and stored at room temperature until required for DNA extractions. Total DNA was extracted from each rod using the methods developed by Billones-Baaijens *et al.* (2018) for spore tape samples.

All DNA extracted from the rods was analysed by nested-PCR and multi-species qPCR assays developed for ED and BD for SAR 1205 (Billones-Baaijens *et al.* 2018, Sosnowski *et al.* 2017). All DNA samples were initially tested for the presence or absence of both ED and BD spores by nested-PCR followed by qPCR for ED and BD pathogens using the methods described in Sosnowski *et al.* 2017 (SAR 1205).

6.2.3 Results

6.2.3.1 Variation around vineyard block

Both ED and BD pathogen spores were detected in the rotorod spore traps for the three consecutive runs over 13-14 August 2020. ED pathogen spores were detected in 17 out of 33 samples, ranging from 5 - 911 spores (Figure 6.25). Spores were detected at all five positions. The greatest number of spores was detected at position 2 (southern edge of vineyard; 1415 spores), with similar numbers detected at positions 1, 3 and 5 (556-664 spores), and most detections occurring in samples from the third run. In general, rotorods positioned at a height of 110 cm, directly above the cordon recorded the greatest number of spores, apart from position 3 (middle of the vineyard), where the greatest number of spores were detected at 40 cm, which is approximately the height of a Burkard volumetric air intake orifice. Also of note, spores were detected in the traps at position 1 (50 m south of the vineyard) during the first (151 pores) and third (513 spores) runs.

BD pathogen spores were detected in five of 33 samples, ranging from 17 to 50 spores (Figure 6.25). The greatest number of spores was detected at positions 1 and 2, on the southern side of the vineyard.

There was 0.8 mm rainfall recorded in the morning of the day prior to the experiment commencing, and then a further 0.8 mm recorded 2 h before the third run commenced. Wind was recorded at an average speed of 3.4 and 4.2 km/h for runs 1 and 2, respectively, both from a NW direction. For the third run, wind speed was recorded at an average of 0.02 km/h from a NE direction.

Eutypa dieback				
1. 50 m away south				
Height	Run 1	Run 2	Run 3	Total
110 cm	0	0	513	513
40 cm	151	0	0	151
Total	151	0	513	664
2. Southern edge				
	Run 1	Run 2	Run 3	Total
110 cm	0	0	504	504
40 cm	0	0	911	911
Total	0	0	1415	1415
3. Middle				
Height	Run 1	Run 2	Run 3	Total
250 cm	0	89	32	121
190 cm	0	0	0	0
110 cm	0	0	498	498
40 cm	62	31	0	93
Total	62	120	530	712
4. Northern edge				
	Run 1	Run 2	Run 3	Total
110 cm	52	58	109	219
40 cm	m	m	m	m
5. Eastern edge				
	Run 1	Run 2	Run 3	Total
110 cm	35	5	403	443
40 cm	52	0	61	113
Total	87	5	464	556

Botryosphaeria dieback				
1. 50 m away south				
Height	Run 1	Run 2	Run 3	Total
110 cm	0	17	0	17
40 cm	50	0	0	50
Total	50	17	0	67
2. Southern edge				
	Run 1	Run 2	Run 3	Total
110 cm	0	0	17	17
40 cm	0	0	25	25
Total	0	0	42	42
3. Middle				
	Run 1	Run 2	Run 3	Total
250 cm	0	0	0	0
190 cm	0	0	0	0
110 cm	0	0	0	0
40 cm	0	0	0	0
Total	0	0	0	0
4. Northern edge				
	Run 1	Run 2	Run 3	Total
110 cm	17	0	0	17
40 cm	m	m	m	m
5. Eastern edge				
	Run 1	Run 2	Run 3	Total
110 cm	0	0	0	0
40 cm	0	0	0	0
Total	0	0	0	0

Figure 6.23. Number of Eutypa and Botryosphaeria dieback pathogen spores detected on a single rod from each rotorod trap attached to a post at one to four heights (40, 110, 190 and 250 cm). Posts with rotorod traps were placed at five different locations in and around the vineyard (Figure 6.21a). The experiment consisted of three runs during August 2020, each with a duration of three hours. Cells are coloured to form heat maps indicating the number of spores detected from low (green) to high (red).

6.2.3.2 Sampling duration

Both ED and BD pathogen spores were detected in the rotorod spore traps over the four consecutive runs from 17-21 August. ED pathogen spores were detected in 12 of the 24 samples, ranging from 37 to 1393 spores (Figure 6.26). Most of the spore detections were during the second and third runs, with the longest sampling duration of 23 h yielding considerably more spores than any other sampling duration during both runs. BD pathogen spores were detected in five of the 24 samples, ranging from 17 to 76 spores. BD spores were detected in the second and third 23 h duration samples, and sporadically at 3, 6 and 18 h across the first and third runs, with no detection in the fourth run. Most rain fell during the second and fourth runs.

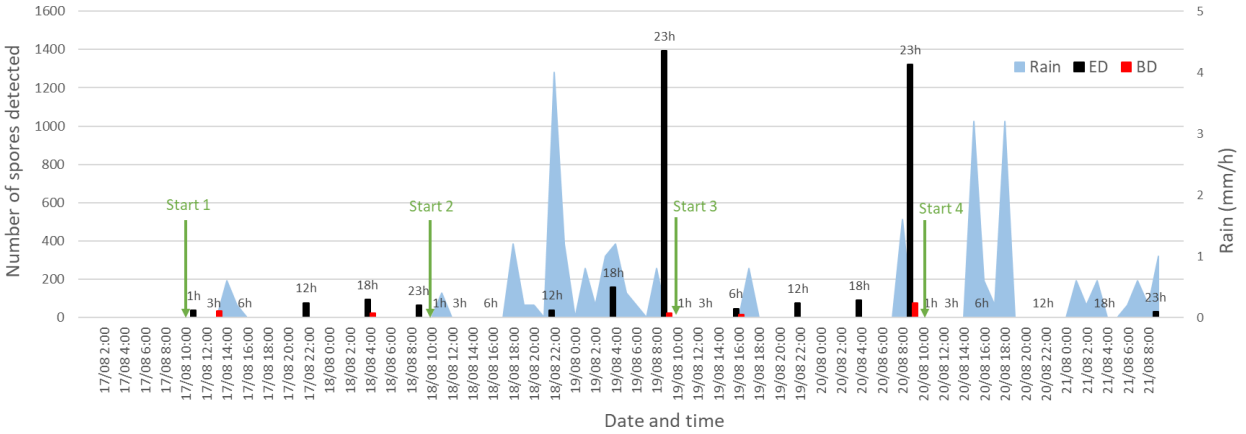


Figure 6.24. Number of Eutypa (ED, black columns) and Botryosphaeria dieback (BD, red columns) pathogen spores detected on rods for sampling durations ranging between 1 and 23 h. Four runs were conducted during August 2020, each starting at 10 am, and hourly rainfall is indicated by the blue shaded area.

6.2.3.3 Monitoring spores in a remediated vineyard

ED pathogen spores were detected at various quantities in the rotorod spore traps over the six consecutive runs from 11-23 August 2021 (Figure 6.27). ED pathogen spores were detected in 64 out of 66 samples, ranging from 103 to 63,417 spores. Overall, at 110 cm above ground, twice the number of spores was detected in the control rows (151,940) compared with the remediated rows (71,880). At 40 cm above ground, more than twice the number of spores was detected in the control rows (189,550) compared with the remediated rows (83,437), and only two positions were represented in the controls, compared with three in the remediated. More spores were detected at the lower height of 40 cm, than at 110 cm. The greatest detection of spores occurred in Runs 5 and 1, which coincided with the highest average wind speeds and the presence of rainfall.

	Eutypa dieback										
		Height	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Sum	Total	
Reworked	Pos 1	40	10938	1133	1304	4084	24428	533	42420		
		110	7038	1618	1459	3152	27144	339	40751		
	Pos 2	40	4982	1391	897	3368	17234	103	27975		
		110	3441	1295	768	2099	12266	198	20066		
	Pos 3	40	0	2034	780	2922	6764	541	13042	83437	40 cm
		110	3778	1989	697	677	3666	257	11063	71880	110 cm
Control	Pos 4	40	34690	2089	1920	6446	55578	634	101357		
		110	22846	1720	1527	3606	3879	591	34169		
	Pos 5	40	17120	1506	1072	3822	63417	1256	88192		
		110	17778	2598	1612	0	21962	na	43951		
	Pos 6	40	na	na	na	na	na	na	na	189550	40 cm
		110	12928	3487	1671	2181	52930	624	73821	151940	110 cm
Total			135539	20861	13706	32357	289270	5075	496807		
	Start date		11-Aug	12-Aug	20-Aug	21-Aug	22-Aug	23-Aug			
	Raifall (mm)		3.6	0.6	0	0	1	2.2			
	wind (km/h)		11.5	5.4	5.7	3.8	11.7	4.5			
	wind dir (deg)		254.5	249.5	269.4	255.7	240.4	168.2			
	wind from		WSW	WSW	W	WSW	SW	S			
			*0.2 mm rain fell on each of 14, 16 and 17 Aug								

Figure 6.25. Number of eutypa dieback pathogen spores detected on a single rod from each rotorod trap attached to a post at one of two heights (40 and 110 cm; Figure 6.24). Posts with rotorod traps were placed at six different positions within the vineyard. The experiment consisted of six runs during August 2021, each with a duration of 24 hours. Cells are coloured to form heat maps indicating the number of spores detected from low (green) to high (red).

BD pathogen spores were also detected at varying quantities in the rotorod spore traps over the six consecutive runs from 11-23 August 2021 (Figure 6.28). BD pathogen spores were detected in 29 out of 66 samples, ranging from 8 - 52 spores. Overall, at 110 cm above ground, 50% more spores were detected in the control rows (546) compared with the remediated rows (365). At 40 cm above ground, six times the number of spores was detected in the control rows (365) compared with the remediated rows (66), and only two positions were represented in the controls, compared with three in the remediated rows. Fewer spores were detected at the lower height of 40 cm, than at 110 cm. The greatest detection of spores occurred in Run 3, which coincided with low wind speeds and no rainfall, other than 0.2 mm recorded 3, 4 and 6 days earlier.

	Botryosphaeria dieback										
		Height	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Sum	Total	
Reworked	Pos 1	40	13	0	0	0	18	0	31		
		110	0	16	52	0	0	0	68		
	Pos 2	40	8	0	0	0	0	0	8		
		110	0	0	128	0	0	11	139		
	Pos 3	40	0	8	0	0	9	10	27	66	40 cm
		110	0	0	135	0	23	0	158	365	110 cm
Control	Pos 4	40	78	32	152	0	8	0	269		
		110	90	0	8	0	0	9	107		
	Pos 5	40	26	0	52	0	18	0	96		
		110	47	8	0	0	10	na	64		
	Pos 6	40	na	na	na	na	na	na	na	365	40 cm
		110	41	143	114	77	0	0	375	546	110 cm
Total			303	206	641	77	86	30	1342		
	Start date		11-Aug	12-Aug	20-Aug	21-Aug	22-Aug	23-Aug			
	Raifall (mm)		3.6	0.6	0	0	1	2.2			
	wind (km/h)		11.5	5.4	5.7	3.8	11.7	4.5			
	wind dir (deg)		254.5	249.5	269.4	255.7	240.4	168.2			
	wind from		WSW	WSW	W	WSW	SW	S			
			*0.2 mm rain fell on each of 14, 16 and 17 Aug								

Figure 6.26. Number of Botryosphaeria dieback pathogen spores detected on a single rod from each rotorod trap attached to a post at one of two heights (40 and 110 cm; Figure 6.24). Posts with rotorod traps were placed at six different positions within the vineyard. The experiment consisted of six runs during August 2021, each with a duration of 24 hours. Cells are coloured to form heat maps indicating the number of spores detected from low (green) to high (red).

There was 3.6, 0.6, 1 and 2.2 mm of rainfall recorded on 11, 12, 22 and 23 August, respectively, and a further 0.2 mm recorded on each of 14, 16 and 17 August, which fell between runs 2 and 3. Wind was recorded at an average speed of 11.5 and 11.7 km/h for runs 1 and 5, respectively, both from a WSW direction. Each of the other runs had average wind speeds of 3.8-5.7 km/h, from the west and south.

6.2.4 Discussion

The rotorod spore traps were able to detect spores of ED and BD pathogens. More ED pathogen spores were detected than BD spores. This is not unexpected, as spores of ED pathogens were also detected in much greater numbers than BD pathogens in a Burkard volumetric spore trap located 24 km away from the Urrbrae vineyard near Hahndorf in the Adelaide Hills and another located at the Nuriootpa Research Centre, located 10 km away from the Greenock vineyard (Section 6.1.3.1). The distribution of ED and BD pathogens is known to vary between Australian wine regions (Pitt *et al*. 2010, Trouillas *et al*. 2011). There was also detailed discussion about the difference in spore size and mode of discharge and their potential impact on spore dispersal and detection (Section 6.1.4). It is likely that ED is the more prevalent disease in the Adelaide Hills and Barossa Valley, which is also supported by the predominance of ED foliar symptoms in these regions that are only associated with *E. lata* (Sosnowski *et al.* 2007b).

The first experiment investigated spore detection at different positions in and around a 27-year-old Shiraz vineyard that showed considerable symptoms of dieback. Most ED pathogen spores were detected in the third run, which followed 0.8 mm of rainfall a few hours before the run commenced. The previous recorded rainfall to this was more than 24 h before the first run commenced. Rain has long been understood to initiate spore release (Carter 1991), and spore trapping studies in the current project have confirmed that most spores are detected following as little as 0.2 mm of rainfall (Section 6.1.3.2). Pathogen spores of ED were detected at all five positions, with an increasing gradient of spore numbers detected from traps located in the north-east corner (positions 4 and 5) to the greatest numbers detected in traps at the southern edge of the vineyard (Position 2). This is consistent with wind recorded predominantly coming from the north during the experiment. Spores were also detected in a trap located 50 m south of the vineyard (Position 1), indicating that inoculum may have been blown from the vineyard. There are also two rows of Dutch elm (*Ulumus* x *hollandica*) trees, planted in 1928, between the vineyard and spore trap at position 1 (Figure 6.21a). *Ulmus* species have been reported as hosts of *E. lata* (Carter 1991) and may further contribute to the number of spores detected at position 1.

Spores of ED pathogens were detected at all heights tested from 40-250 cm. The greatest number of spores was generally detected at the height of 110 cm, which represented the height of the pruning wound zone of the cordon-pruned vines. ED spores were often also detected at 40 cm, although at lower numbers, which represents the height of the air intake orifice of the Burkard volumetric spore trap. This suggests that data from the Burkard spore traps are representative but may underestimate the number of spores present at the height of cordon pruning wound zone.

Detection of BD spores was very low in this experiment, and almost all of the spores were detected at positions 1 and 2, and no spores detected at positions 3 and 5. Due to the low numbers, little can be concluded about the distribution of BD pathogen spores.

The second experiment evaluated the duration of spore collection in rotorod traps. It revealed that the greatest number of ED pathogen spores was detected when the traps were run for 23 h, the longest duration tested. However, in the first and fourth runs, the 23 h duration did not increase in spore numbers compared to shorter durations. In the first run there was only 0.8 mm rain, compared with 11.8 mm during the second run, which may be why more spores were detected in the latter. The lower number of spores detected in the fourth run is consistent with a previous report by Dubos (1987; cited by Carter 1991) that *E. lata* spore liberation began 2 h after rainfall commenced and then ceased more than 36 h later, even with continual rain. The authors reported that about 12 days was required for the development of new spores ready to be discharged from fungal fruiting bodies.

Again, BD pathogens were only sporadically detected in low numbers in the first three runs, and hence no conclusions can be made about optimal run duration.

In the third experiment in the Barossa Valley, both ED and BD pathogens were detected in much greater numbers in the control vines, compared to the remediated vines. Visual inspection of the control vines revealed dead arms with cankers that would likely harbour fruiting bodies of both pathogens, providing a source of new inoculum each time rainfall occurs. The lower spore numbers detected in remediated vines suggests that although spores are airborne, and in the case of *E. lata* reported to travel long distances of up to 160 km (Moller and Carter 1965), they are most likely deposited in greater numbers when closer to the source. The adjacent block directly to the west of the remediated block also had vines with dieback that were not remediated, so it is most likely that some spores were blown from this source during the predominantly westerly winds. Further research using rotorod spore traps could monitor spores at different distances from a source, when isolated from other sources, to determine the gradient of spore dispersal.

Results from the third experiment also revealed that greater spore numbers were detected at the lower height of 40 cm for ED pathogens, contrary to the result in the first experiment in 2020 at Urrbrae. Further, in the third experiment, greater BD pathogen spore numbers were detected at 110 cm. This shows the variability associated with spore monitoring and the need for repeated experiments at different times and locations.

In terms of disease management, although remedial surgery may reduce the level of inoculum and infection risk within the vineyard, annual wound protection is still necessary to prevent infection from spores derived from inoculum sources outside of the vineyard. Rotorod spore traps provide an effective research tool for monitoring spore dispersal in vineyards under different management regimes.

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7 PRUNING WOUND SUSCEPTIBILITY

7.1 Introduction

Numerous international studies have been conducted into the susceptibility of grapevine pruning wounds to infection by grapevine trunk disease pathogens. Research conducted in Australia on apricots 50 years ago (Carter and Moller 1967, 1970) reported that wounds were susceptible to infection by the Eutypa dieback pathogen *Eutypa lata* for up to four weeks during early winter, with decreased susceptibility in late winter and early spring. Pruning wounds were reported to be more susceptible to *E. lata,* and for a longer duration, in early winter than in spring in California (Moller and Kasimatis 1980, Petzoldt *et al.* 1981 and Munkvold and Marois 1995), whereas wounds were reported with higher susceptibility to *E. lata* in late winter and early spring than in early winter in south-west France (Chapius *et al*. 1998), Michigan (Trese *et al.* 1982), and South Africa (van Niekerk *et al*. 2011). Serra *et al*. (2008) reported that, in Italy, wounds were susceptible to infection by Botryosphaeria dieback pathogens for up to 16 weeks with no difference between winter and spring pruning times, while in California, Úrbez-Torres and Gubler (2011) reported a wound susceptibility duration of 12 weeks with decreased susceptibility in late winter and early spring compared with early winter, and in South Africa, van Niekerk *et al.* (2011) reported a susceptibility duration of 21 days or more with greatest susceptibility when pruned in late winter compared with early winter. More recently, comprehensive research trials were conducted as part of Wine Australia funded project SAR 1205, which examined the susceptibility of grapevine pruning wounds to infection by *E. lata* in the warm-dry climate of McLaren Vale, SA and the Botryosphaeria dieback pathogens *Diplodia seriata* and *Neofusicoccum luteum* in the hot-dry climate of Wagga Wagga, NSW according to Dry and Coombe (2008). Wounds were highly susceptible to infection immediately following pruning, and susceptibility decreased sharply over the following two weeks, and at varying rates, depending on the pathogen being evaluated, the year of the trial, and the region (Ayres *et al*. 2016, Sosnowski *et al*. 2017).

These reports from different regions around the world indicate that there is variation in the duration of susceptibility of grapevine pruning wounds to infection by trunk disease pathogens at different times during the pruning season. Localised research is required to gain an understanding of the periods of greatest wound susceptibility in different climatic regions. This will allow growers to better target their wound protection strategies to times of greatest need. Therefore, research has extended pruning wound susceptibility evaluation to the warm-wet region of Adelaide Hills SA (Dry and Coombe 2008).

Canopy management by shoot thinning during the spring by trimming green shoots or de-suckering is undertaken to improve airflow for reduced disease, increase leaf exposure to sunlight for increased photosynthesis and assist with maintenance of crop yield and quality, and also to reduce the number of pruning wounds in the following winter (Smart *et al.* 1990, Smart and Robinson 1991, Smart 1992, Coombe and Illand 2004). Recent spore trapping research in Australian vineyards has revealed that spores of Eutypa and Botryosphaeria dieback pathogens are often detected during spring in association with rainfall in all Australian wine regions that have been monitored (Billones-Baaijens *et al.* 2017, Sosnowski *et al*. 2017, SAR 1205, Chapter 5). It is well documented that Botryosphaeria dieback pathogens can infect green grapevine tissue (Úrbez-Torres 2011, Amponsah et al. 2012) but there is no evidence of green shoot infection by Eutypa dieback pathogens, other than one report from research conducted by Trouillas and Gubler (2010a) which involved mycelial rather than spore inoculum. Therefore, the risk of infection by grapevine trunk disease pathogens during spring shoot thinning is not fully understood and requires investigation.

7.2 Methods

7.2.1 Winter pruning

A vineyard trial was established in 2017 on cv. Shiraz vines planted in 1997 near Hahndorf, in the Adelaide Hills, SA.

One-year-old canes were pruned to 3-4 buds on 8 June, 20 July and 31 August in 2017 using secateurs (Figure 7.1a). For each pruning time, wounds were inoculated at 1, 7, 14, 21, 28, and 42 days following pruning for *E. lata*, and at 1, 7, 14, 28, 42, 56 and 84 days for *D. seriata* (Botryosphaeria dieback). A non-inoculated control (NIC) was also included at each pruning time to monitor natural infection. At each inoculation time, wounds were moistened by spraying with sterile distilled water (SDW) immediately prior to inoculation with approximately 100 or 200 spores of *E. lata* or *D. seriata* suspended in 20 µL SDW and applied using a pipette (Figure 7.1b). Two inoculum doses were used for each pathogen to maximise the chance of having adequate recovery from wounds at one-day post-pruning, and to compare pruning and inoculation times. Each treatment was allocated to a vine with 10 pruned canes. The trial was set up as a randomised block design with five replications. The trial was repeated in 2018 with pruning occurring on 7 June, 19 July and 30 August and again in 2019 with pruning on 6 June, 18 July and 29 August.



Figure 7.1. Methods used to evaluate wound susceptibility in the vineyard: (a) cutting canes to 3-4 bud spurs on Shiraz vines near Hahndorf in the Adelaide Hills, (b) inoculating wounds with ascospores of *Eutypa lata*, (c) spurs with bark removed, (d) cutting chips from segments that spanned the interface between normal and stained wood and (e) cultures of *E. lata* and (f) *Diplodia seriata* growing from wood chips.

Eutypa lata ascospores were obtained from fruiting bodies, using methods similar to those described by Carter (1991). This involved attaching pieces of dead grapevine wood containing perithecia to a plastic lid and soaking them in a container of water for one hour. The lid, with wood attached, was then placed onto a clean plastic container and left overnight. The following day, spores were washed with water from the sides of the container into a suspension and adjusted to 10,000 spores/mL using a haemocytometer.

Diplodia seriata was grown from storage on petri dishes containing potato dextrose agar (PDA) amended with antibiotic (streptomycin sulfate 100 mg/L) incubated at 25°C under fluorescent light with 12 h photoperiod for 3-4 weeks. Spores were washed with SDW from the plates and the concentration adjusted to 10,000 spores/mL using a haemocytometer.

For each pathogen, spore germination was assessed by placing four drops of the spore suspension on a PDA plate, which was then placed in an incubator at 25°C under fluorescent light with 12 h photoperiod. After 12-24 hrs, using a compound microscope at 200x magnification, the number of non-germinated spores per 100 in each of the drops was counted and a mean percentage of germinated spores was determined.

Canes were removed from vines in early May the following year, up to 11 months after trial establishment, and returned to the laboratory for assessment. Bark was removed from each cane using a sharp knife (Figure 7.1c). The exposed wood was surface disinfested in 2.5% sodium hypochlorite (NaOCl) for 10 minutes and then washed twice in SDW. Using flame-disinfested secateurs, canes were cut into segments ($3 \times 2 \times 2$ mm) that spanned the interface between normal and stained wood tissue (Figure 7.1d). Five wood segments were plated in plastic Petri dishes filled with PDA amended with antibiotic (streptomycin sulfate 100 mg/L), with two plates used for each cane. Samples were incubated at 25°C under fluorescent light for 12 h photoperiod for seven days and then assessed for the presence or absence of mycelial growth of *E. lata* or *D. seriata* (Figure 7.1e-f). Data were subjected to analysis of variance and least significant difference (LSD) at the 5% level was used for all pair-wise comparisons; standard error of the means were calculated.

Climate data were provided by the Adelaide and Mount Lofty Ranges Natural Resources Management Board, via the Adelaide Hills Wine Region industry association.

7.2.2 Spring pruning

A preliminary trial was established in a shade house at the Waite Campus in Urrbrae SA to evaluate the potential for infection by Eutypa and Botryosphaeria dieback pathogens during spring shoot thinning. In winter 2019, cuttings of cv. Shiraz were collected from the Nuriootpa Research Centre and in the following spring were propagated by cutting to 3-4 nodes (30-40 cm) which were then placed in trays filled with perlite/ vermiculite mix and maintained in a greenhouse at 15-30°C. Following callusing and root development, rootlings were transferred to 20 cm pots filled with potting soil and placed in a shade house. In winter 2020 all vines were pruned to leave 6-8 buds per vine.

On 9 November 2020, shoots that were 30-50 cm long were either cut with secateurs about 1 cm above the second or third node leaving a smooth pruning wound (Figure 6.2a), or the whole green shoot was torn off at the joint between lignified cane and the base of the shoot, leaving a rough socket wound (Figure 6.2b). Wounds were inoculated as described in Section 6.2.1. Briefly, wounds were moistened by spraying with sterile distilled water (SDW) immediately prior to inoculation with approximately 200 spores of *E. lata* or *D. seriata* suspended in 20 µL SDW and applied using a pipette (Figure 6.2c-d). In general, on each vine, two spurs were allocated to a pathogen each, and each spur was subjected to both types of wounds (Figure 6.2e). The trial was set up as a completely randomised design with 50 replications set up on a bench, and on five extra vines a further 10 pruning wounds and 10 socket wounds were left uninoculated as controls (Figure 6.2f).



Figure 7.2. Spring pruning trial in the shade house, showing (a) smooth pruning wound above the node, (b) rough socket wound at base of green shoot, (c-d) inoculating the wounds with pathogen spores, (e) both types of wounds on a single spur and (f) bench with all vines in pots containing the 50 tagged spurs for each of the Eutypa (orange) Botryosphaeria (blue) dieback pathogens.

Spurs were removed from vines on 2 August 2021, and returned to the laboratory for assessment as described in Section 6.2.1. Briefly, bark was removed from spurs around wound sites before surface sterilising in 2.5% sodium hypochlorite (NaOCl) for two minutes and then washed twice in SDW. Canes were cut into small segments that were placed on PDA plates. Samples were incubated for seven days and assessed for presence or absence of *E. lata* or *D. seriata*. The percentage of pathogen recovery from wounds was calculated and graphed in Microsoft Excel.

7.3 Results

7.3.1 Winter pruning

7.3.1.1 Eutypa dieback

Based on preliminary assessments of subsamples from one-day inoculation treatments, all treatments inoculated with *E. lata* spore dosages of 200 were assessed in 2018, 2019 and 2020, as these provided adequate incidence of recovery. Remaining samples from the 100 spore doses in each year were discarded. For the *E. lata* inocula, the average spore germination was 89, 92 and 94% for 2017, 2018 and 2019, respectively.

In 2017-18 mean recovery from wounds inoculated with *E. lata* was significantly lower for the vines pruned in late August, (36% recovery when inoculated one-day post-pruning) than for vines pruned in early June (100%) and mid-July (89%; Figure 7.3). For the early pruning time, mean recovery of *E. lata* was reduced significantly to 4% when inoculated 14 days post-pruning, and for the mid and late pruning times, to 14 and 20%, respectively, when inoculated seven days post-pruning. For the early and mid-pruning times, mean recovery rates for wounds inoculated 14 to 42 days post-pruning were similar to those for the NICs (0 and 5%, respectively) and for the late pruning time, recovery rates for wounds inoculated 21-42 days post-pruning were similar to those for NICs (6%).

In 2018-19, mean recovery from wounds inoculated with *E. lata* was significantly lower for the vines pruned in late August (24% recovery when inoculated one-day post-pruning), than for vines pruned in mid-July (46%), which was significantly lower than vines pruned in early June (75%; Figure 7.3). For

the early and mid-pruning times, recovery was significantly reduced to levels similar to that of the NICs for all inoculation times from 7 to 42 days post pruning, while for the late pruning time, there was no significant difference in recovery from wounds between any of the inoculation times and the NICs.

In 2019-20, mean recovery from wounds inoculated with *E. lata* was significantly lower for the vines pruned in late August (57% recovery when inoculated 1-day post-pruning) than for vines pruned in early June (84%; Figure 7.3). Recovery of *E. lata* from vines pruned in mid-July (17% recovery when inoculated 1-day post-pruning) was lower than for vines inoculated seven days post pruning (20% recovery) leading to the conclusion that the day 1 inoculation was compromised. For the early and late pruning times, recovery was significantly reduced to levels similar to that of the NICs for all inoculation times from 14 to 42 days post pruning, while for the mid pruning time, there was no significant difference in recovery from wounds between any of the inoculation times and the NICs (8-15%).

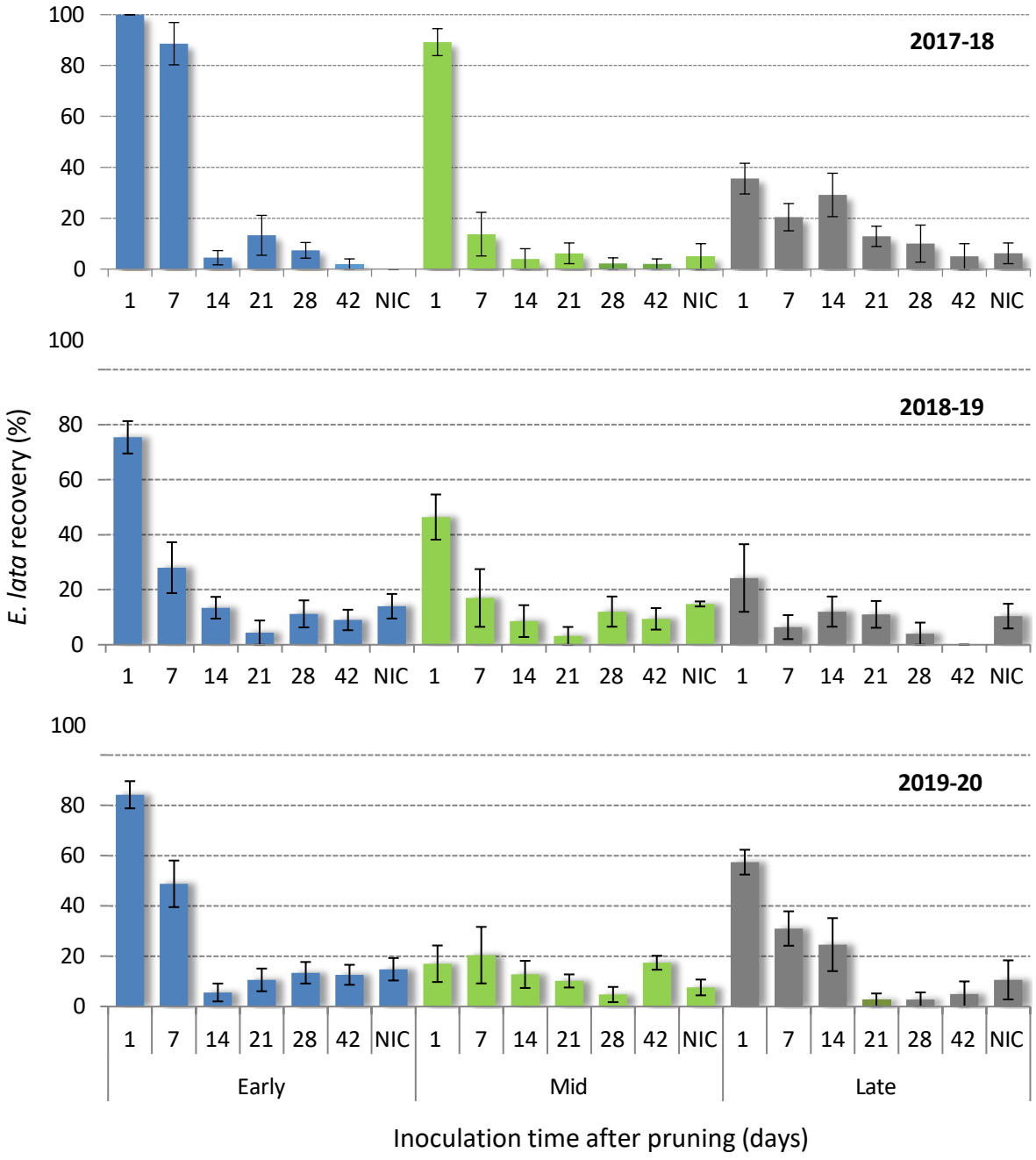


Figure 7.3. Incidence of recovery of *Eutypa lata* in the Adelaide Hills trial from canes pruned on 8, 7 and 6 June (Early), 20, 19 and 18 July (Mid) and 31, 30 and 29 August (Late) in 2017-18, 2018-19 and 2019-20, respectively. Wounds were inoculated with 200 *E. lata* spores at 1, 7, 14, 21, 28 and 42 days after pruning. NIC = non-inoculated control. Bars represent standard error of the mean.

7.3.1.2 Botryosphaeria dieback

Based on preliminary assessments of subsamples from one-day inoculation treatments, all treatments with *D. seriata* spore dosages of 200 were assessed in 2018, 2019 and 2020, as these provided adequate incidence of recovery. Remaining samples from the 100 spore doses in each year were discarded. For the *D. seriata* inocula, the average spore germination was 68, 63 and 62% for 2017, 2018 and 2019, respectively.

In 2017-18, susceptibility of wounds to infection by *D. seriata* was significantly greater in vines pruned in early June (87% recovery when inoculated one-day post-pruning) compared with vines pruned in mid-July (54%) and late August (62%; Figure 7.4). For all three pruning times, recovery was significantly reduced from vines inoculated seven days post pruning (57, 25 and 19% for early, mid and late pruning times, respectively) and further reduced to levels similar to those of the NICs for all inoculation times from 14 to 84 days post pruning (0–10%, 0–9% and 2–3%, respectively).

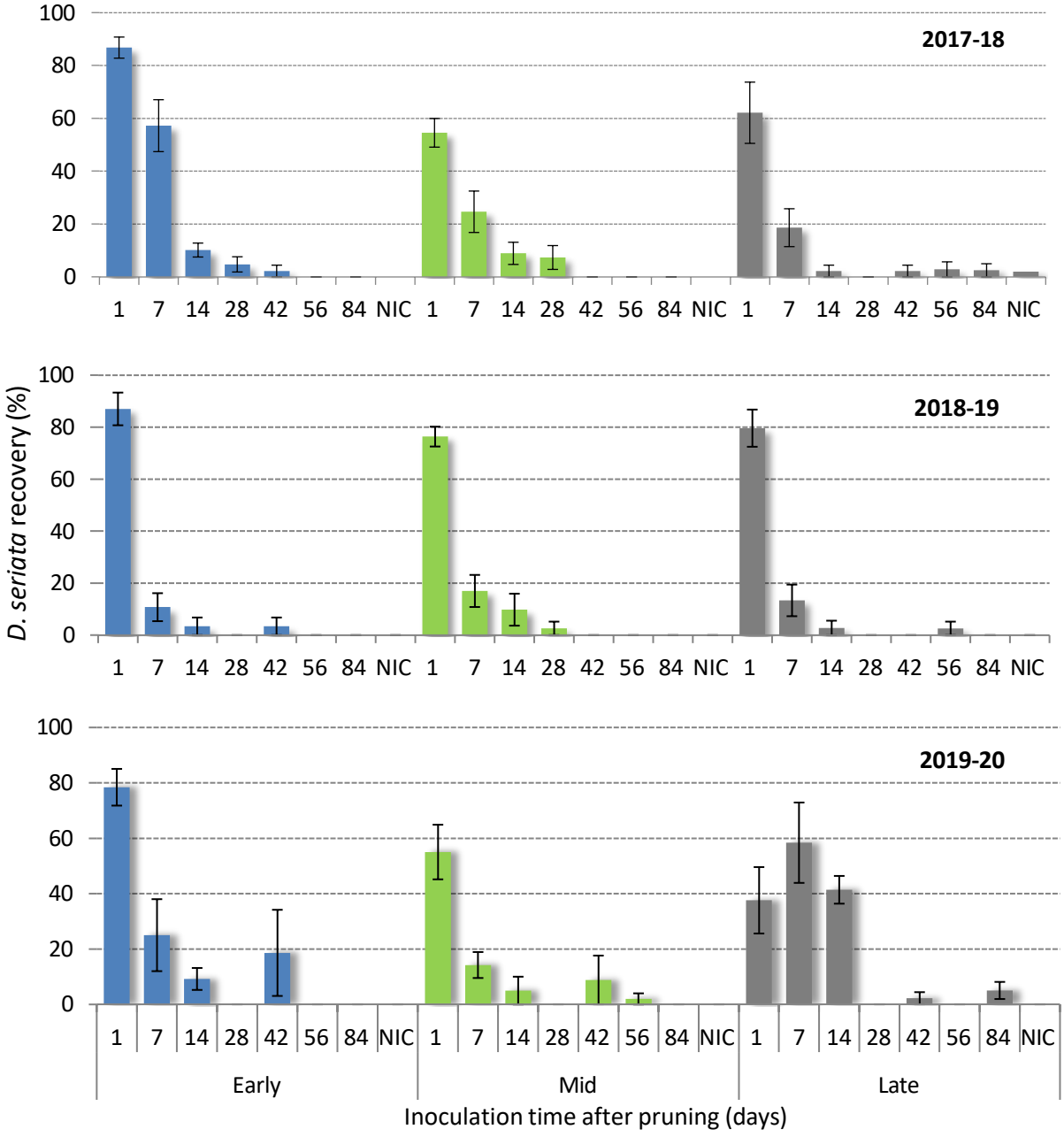


Figure 7.4. Incidence of recovery of *Diplodia seriata* from canes pruned on 8, 7 and 6 June (Early), 20, 19 and 18 July (Mid) and 31, 30 and 29 August (Late) in 2017-18, 2018-19 and 2019-20 respectively. Wounds were inoculated with 200 *D. seriata* spores at 1, 7, 14, 28, 42, 56 and 84 days after pruning. NIC = non-inoculated control. Bars represent standard error of the mean.

In 2018-19, the susceptibility of wounds to infection by *D. seriata* was similar at all pruning times with recovery ranging from 76–87%; Figure 7.4). For early, mid and late pruning times, recovery was significantly reduced for the vines inoculated seven days post pruning (11, 17 and 13%, respectively) and reduced to negligible levels similar to that of the NICs for all inoculation times from 14 to 84 days post pruning (0–3%, 0–9% and 0–3%, respectively).

In 2019-20, susceptibility of wounds to infection by *D. seriata* was significantly greater in vines pruned in early June (78% recovery when inoculated one-day post-pruning) compared with vines pruned in mid- July (55%) and late August (38%), but similar to the late August days 7 and 14 inoculations (58 and 41%; Figure 7.4). For the early and mid-pruning times, recovery was significantly reduced for the vines inoculated seven days post pruning (25 and 14%, respectively) and reduced to levels similar to that of the NICs for all inoculation times from 14 to 84 days post pruning (0–19%, 0–9% and 0–41%, respectively). For the late pruning time, recovery was significantly reduced to levels similar to that of the NICs for all inoculation times from 28 to 84 days post pruning (0–5%).

7.3.1.3 Climate data

For each year, rainfall was scattered throughout May to November, with the highest falls corresponding to the lowest temperatures (Figure 7.5 and 7.6). In 2017, most rainfall occurred in July and August and in 2018 rain was prevalent across June, July and August, whereas in 2019, there was minimal rainfall in July and August. Temperature increased from late August each year. Mean daily maximum temperatures for the 14 days following early, mid and late pruning times were 14.5, 14.2 and 14.8°C (2017), 12.7, 14.2 and 15.6°C (2018) and 13.7, 13.4 and 14.1°C (2019). Total rainfall for the 14 days following each pruning time in each year was 5.8, 39.4 and 83.4 mm (2017), 82.6, 36.4 and 17.2 mm (2018) and 0.4, 0.8, 33.4 mm (2019).

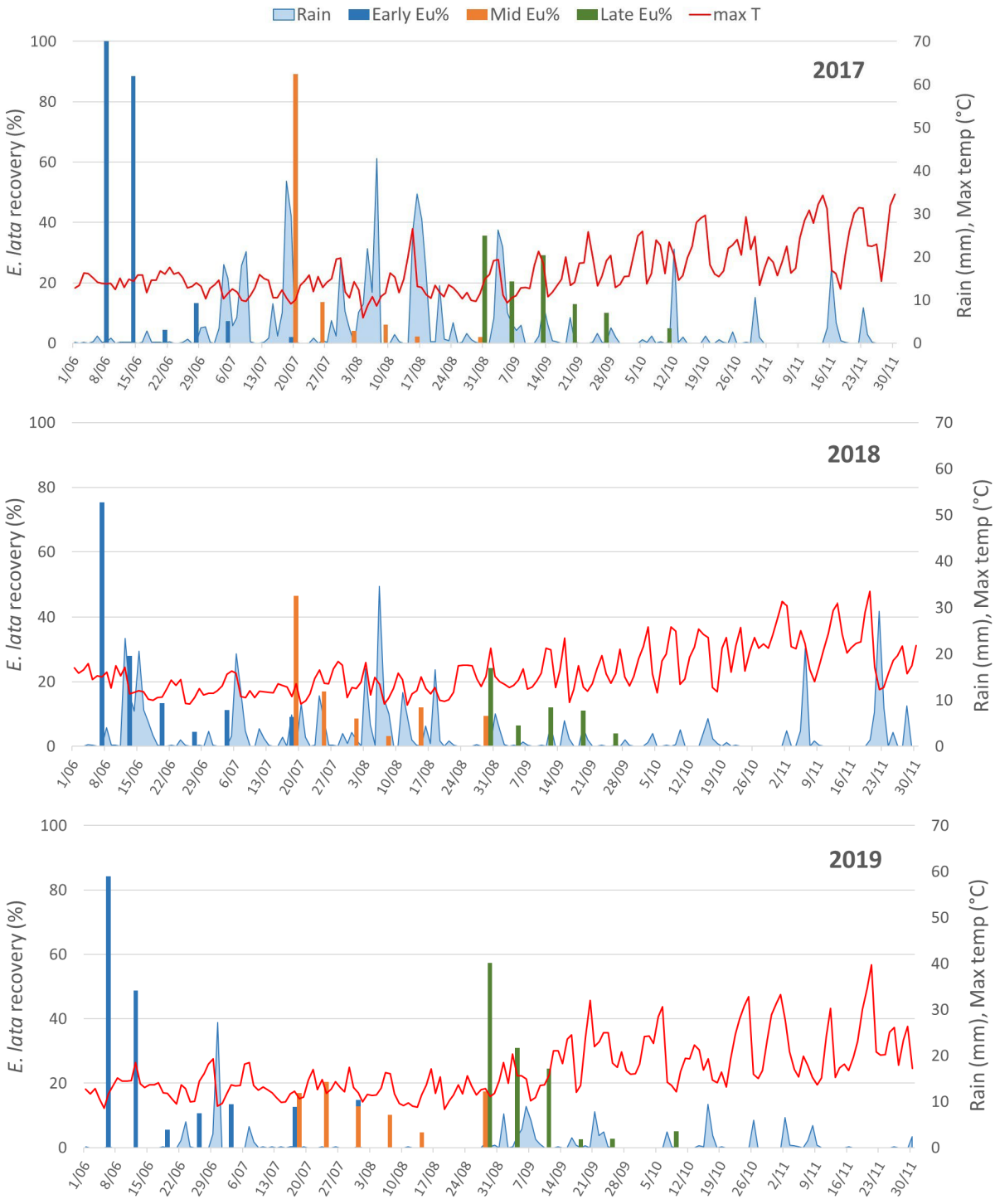


Figure 7.5. Weather data and *Eutypa lata* recovery percentage (solid bars) associated with the six inoculation times (1, 7, 14, 21, 28 and 42 days) following each pruning time in early-June (blue), mid-July (orange) and late-August (green) at the Adelaide Hills trial site for 2017, 2018 and 2019. Values on the right axis are total daily rainfall (mm) and maximum daily temperature (°C).

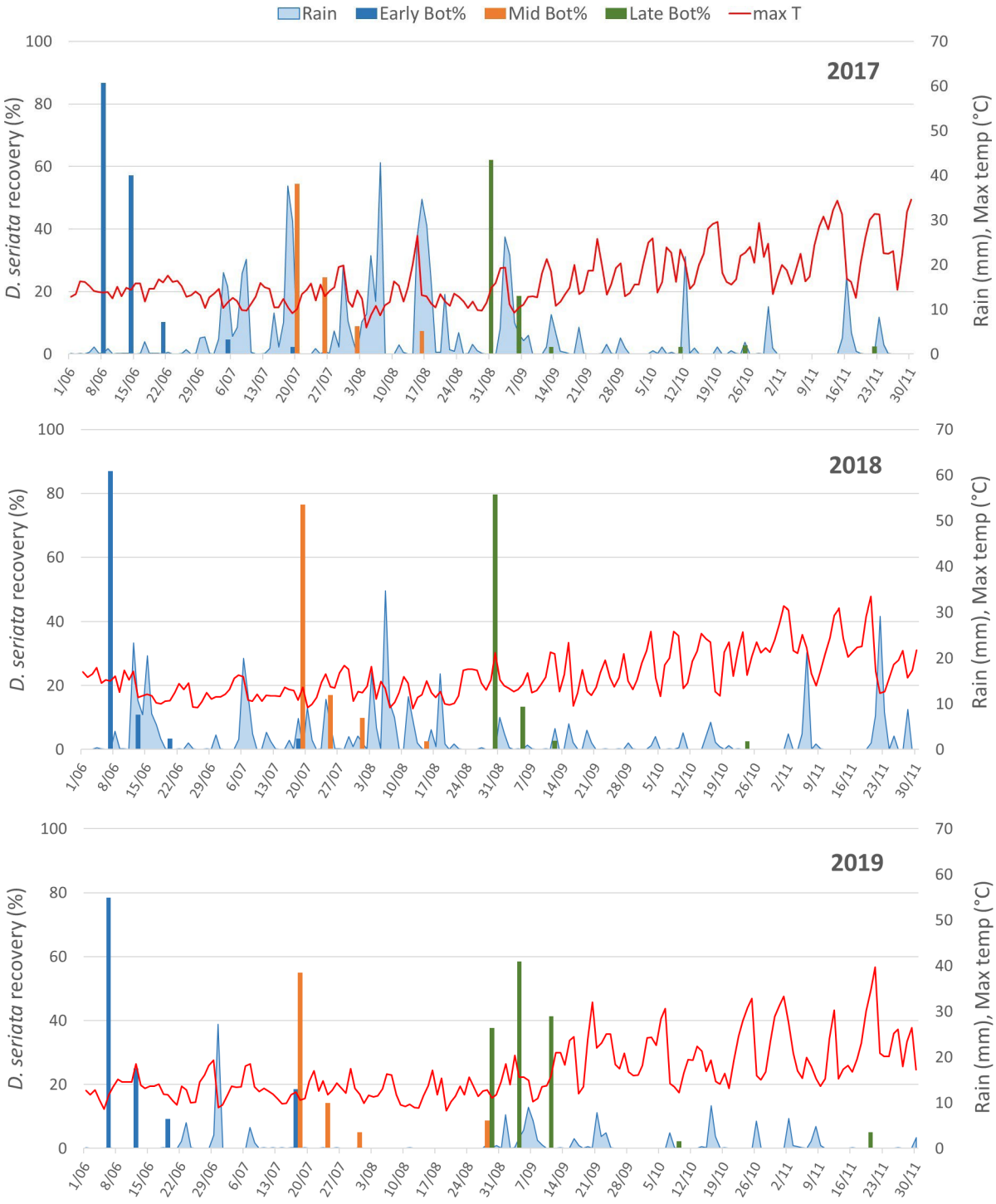


Figure 7.6. Weather data and *Diplodia seriata* recovery percentage (solid bars) associated with the seven inoculation times (1, 7, 14, 28, 42, 56 and 84 days) following each pruning time in early-June (blue), mid-July (orange) and late-August (green) at the Adelaide Hills trial site for 2017, 2018 and 2019. Values on the right axis are total daily rainfall (mm) and maximum daily temperature (°C).

7.3.1.4 Cryptovalsa ampelina

During the assessment of canes for *E. lata* and *D. seriata*, it was observed that plates often also contained other white cultures, which were morphologically different to *E. lata*, and suspected to be a related Diatrypaceaous species (Figure 7.7). Representative cultures were confirmed as *Cryptovalsa ampelina* by PCR using *C. ampelina*-species specific primers developed for SAR 1205. The crude DNA extractions from fungal mycelia and PCR were performed following the methods described in SAR 1205 (Sosnowski *et al*. 2017).

Throughout the assessment of all canes in both experiments, incidence of *C. ampelina* was recorded. For the canes inoculated with *E. lata*, recovery of *C. ampelina* varied between pruning times, particularly in 2017-18, but with no distinct patterns across each of the inoculation times (Figure 7.8). In 2017-18, mean recovery of *C. ampelina* was 53–76% when pruned in mid-July, significantly greater than when pruned in early June (10–34%), with recovery from canes pruned in late August ranging between 25 - 60%. In 2018-19, mean recovery of *C. ampelina* from canes was 19–36% (when pruned early June), 31–65% (mid-July) and 18–47% (late August), with no clear differences between pruning or inoculation times. In 2019-20, mean recovery of *C. ampelina* from canes was 0–10% (early June), 7– 21% (mid-July) and 16–35% (late August), with greater incidence when pruned in late August, compared with early June. There were no clear differences between inoculation times. Recovery of *C. ampelina* from the non-inoculated control canes was similar to that from the inoculated canes.

For the canes that were inoculated with *D. seriata*, recovery of *C. ampelina* was similar to that from the *E. lata* trial. Recovery varied between pruning times, with no distinct patterns across the inoculation times (Figure 7.9). In 2017-18, mean recovery of *C. ampelina* was 42–78% when pruned in mid-July, significantly greater than when pruned in early June (0–11%), with recovery from canes pruned in late August ranging between 25 and 39%. In 2018-19, mean recovery of *C. ampelina* from canes was 18–40% (when pruned early June), 38–71% (mid-July) and 26–44% (late August), with greatest incidence when pruned in mid-June. In 2019-20, mean recovery of *C. ampelina* from canes was 5–18% (early June), 5–22% (mid-July) and 21–43% (late August) with greater incidence when pruned in late August, compared with early June and mid-July.

At the time of pruning the vines in June 2018, 20 randomly selected canes (20 cm long extending from the pruning cut) were kept from the trial site and returned to the laboratory for isolation onto agar plates, as described above. Relatively few organisms were recovered from the wounds of canes, with no *C. ampelina*, *E. lata* or *D. seriata* recovered.

To further investigate the increased presence of *C. ampelina*, diseased grapevine wood was collected from trial vineyards in McLaren Vale and Clare Valley regions in SA. Under a dissecting microscope (5x magnification), perithecia (fruiting bodies) were identified and sliced open with a scalpel. A water droplet was placed on the exposed fruiting body to liberate asci (spore sacs), which were transferred to microscope slides and viewed under a compound microscope (200x magnification) to count ascospores. Asci commonly contained 16 ascospores (typical of *C. ampelina*) whereas *E. lata* asci only contain eight asci. Spores were single-spore cultured on PDA and morphological identification confirmed the presence of *C. ampelina* cultures.

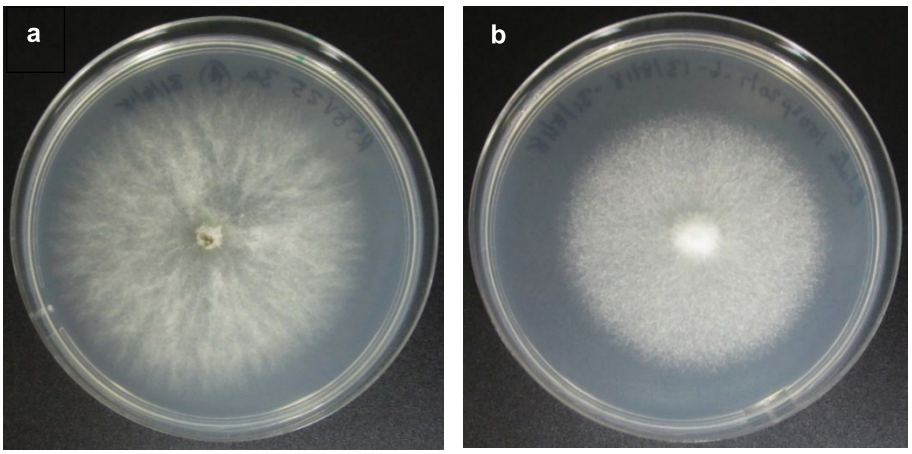


Figure 7.7. Fungi from the Diatrypaceae recovered from canes from the Adelaide Hills susceptibility trial in 2018: (a) *Cryptovalsa ampelina* and (b) *Eutypa lata*.

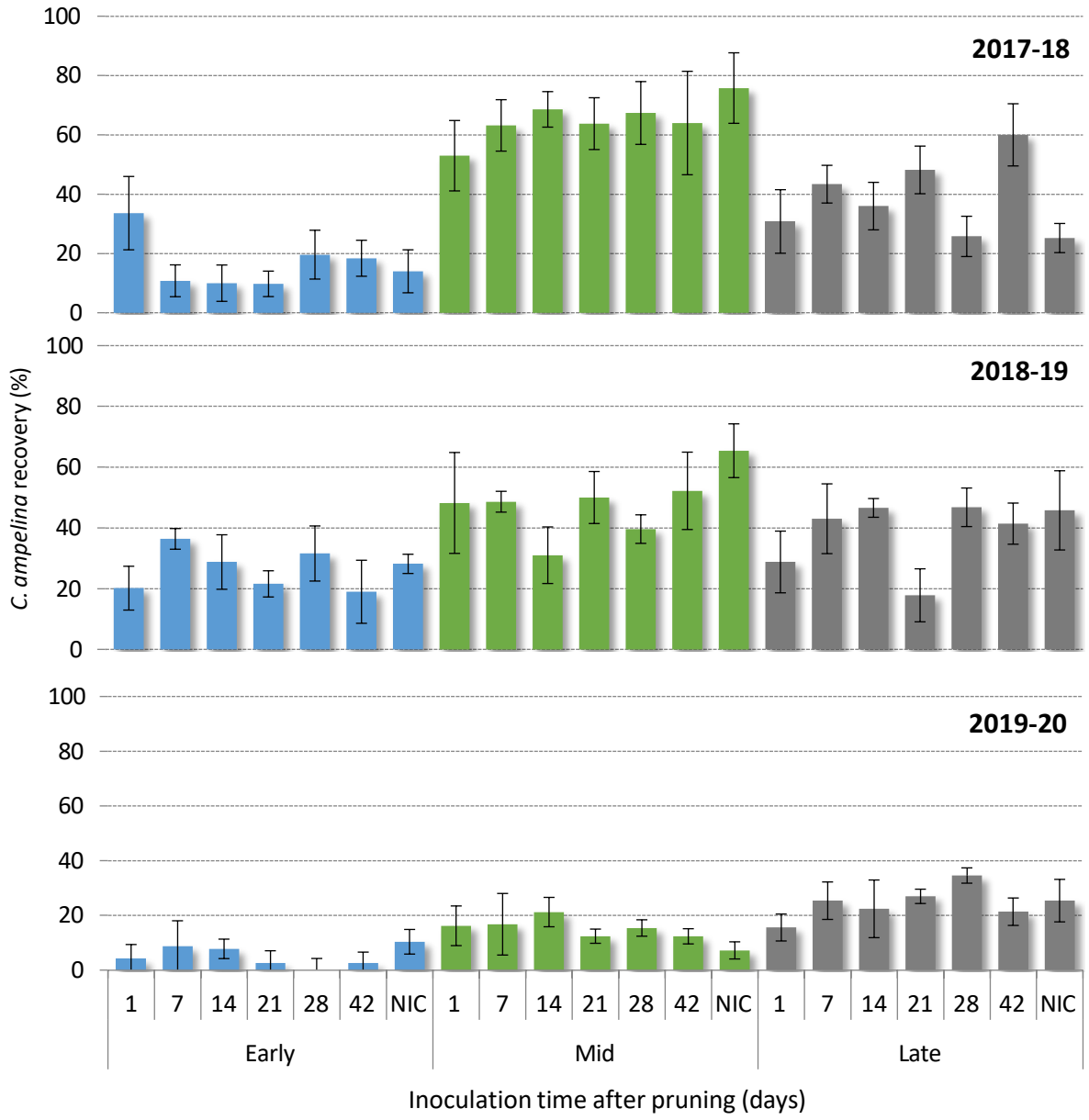


Figure 7.8. Incidence of recovery of *Cryptovalsa ampelina* from canes pruned on 8, 7 and 6 June (Early), 20, 19 and 18 July (Mid) and 31, 30 and 29 August (Late) in 2017-18, 2018-19 and 2019-20, respectively. Wounds were inoculated with 200 *E. lata* spores at 1, 7, 14, 21, 28 and 42 days after pruning. NIC = non-inoculated control. Bars represent standard error of the mean.

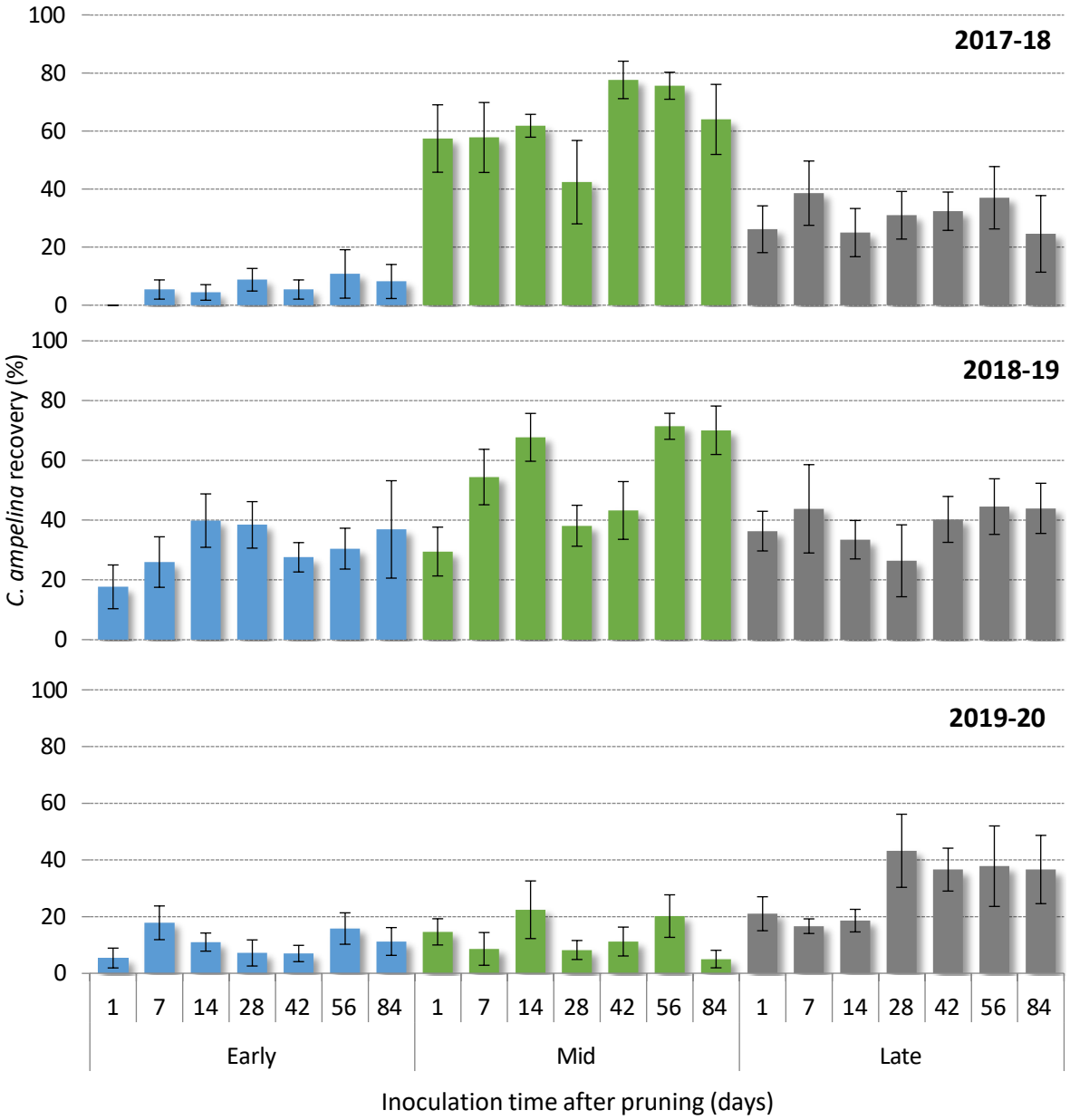


Figure 7.9. Incidence of recovery of *Cryptovalsa ampelina* from canes pruned on 8, 7 and 6 June (Early), 20, 19 and 18 July (Mid) and 31, 30 and 29 August (Late) in 2017-18, 2018-19 and 2019-20, respectively. Wounds were inoculated with 200 *D. seriata* spores at 1, 7, 14, 21, 28, 42 and 56 days after pruning. Bars represent standard error of the mean.

7.3.2 Spring pruning

Pathogens were readily recovered from pruning and socket wounds eight months after inoculation with 200 spores per wound (Figure 7.10). *Eutypa lata* was recovered from 62 and 69% of pruning and socket wounds, respectively. *Diplodia seriata* was recovered from 96 and 91% of pruning and socket wounds, respectively.

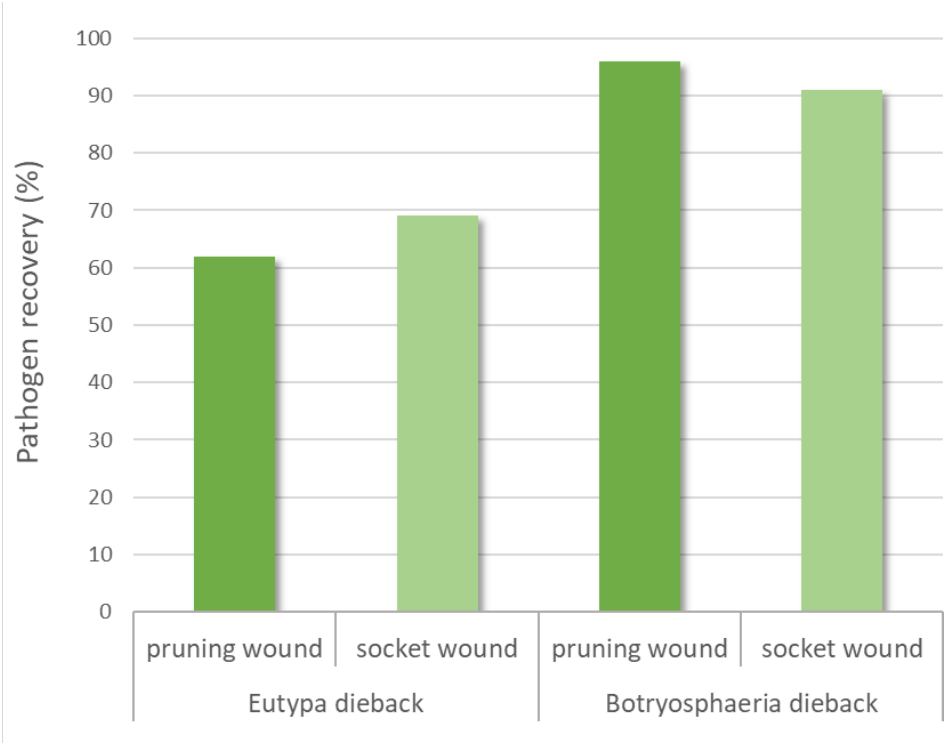


Figure 7.10. Recovery of *Eutypa lata* (Eutypa dieback) and *Diplodia seriata* (Botryosphaeria dieback) from pruning wounds above the second node on green shoots and socket wounds from green shoot removal. Wounds were inoculated with 200 spores of either pathogen.

7.4 Discussion

Pruning wounds were highly susceptible to ED and BD pathogens immediately following pruning, after which the susceptibility decreased rapidly over the following 14 days. From 21 days post-pruning, susceptibility was generally negligible, and often similar to the NICs. This indicates that wounds are most vulnerable to infection for the first 14 days following pruning, similar to that reported for *E. lata* on apricot by Carter and Moller (1970) and more recently reported for *E. lata* and *N. luteum* on grapevines in McLaren Vale and Wagga Wagga, respectively (Ayres *et al.* 2016, Sosnowski *et al.* 2017, SAR1205). However, in Wagga Wagga, *D. seriata* was recovered from wounds for a longer duration following pruning than recorded in the Adelaide Hills. This may be partly due to the very high natural levels of *D. seriata* recorded in the Wagga Wagga trials (up to 35% recovery from NICs) compared with the very low levels recorded in the Adelaide Hills (only 2% on one occasion). Furthermore, the *D. seriata* inoculum dose used in the Adelaide Hills trial was 200 spores, compared with 1000 spores in the Wagga Wagga trial, possibly reflecting lower disease pressure in the Adelaide Hills trial, closer to that of natural infection levels.

Carter and Moller (1971) showed that on apricots, as few as 10 *E. lata* spores would be expected to naturally infect a single pruning wound, so although high spore doses may be necessary to ensure good recovery of the pathogen in an experimental situation, this may lead to an unnaturally high disease pressure, which may also influence the apparent rate of wound healing. Elena *et al.* (2015) reported 8-45% recovery from grapevine wounds inoculated with only 10 spores of either *E. lata* or *D. seriata*, which increased to 90-100% when inoculated with 500 or 1000 spores, respectively. Therefore, the longer duration of susceptibility to infection by BD pathogens reported by Serra *et al.* (2008) and Úrbez-Torres and Gubler (2010) could be due to the high spore doses used, ranging between 2500 and 5000

spores per wound. The differences are unlikely to be due to the different cultivars used, as it has been reported that cultivars do not vary in susceptibility to ED and BD pathogens (Sosnowski *et al.* 2017, SAR 1205). The use of only 200 spores per wound in the current study, although still higher than expected to occur naturally, may help provide a more realistic simulation of wound susceptibility over time.

With regard to wound protection, fungicide timing trials indicated that ED and BD pathogens are controlled when wounds are treated with fungicide up to six days after infection, and will continue to provide control of both pathogens for 1-2 weeks (Ayres *et al.* 2017, 2022). Therefore, a single application of a registered fungicide could provide 2-3 weeks of wound protection, which covers the most susceptible period of two weeks post-pruning.

Pruning wounds were generally most susceptible to infection by *E. lata* when pruned in early June and least susceptible when pruned in late August, supporting reports from California (Moller and Kasimatis 1980, Petzoldt *et al.* 1981 and Munkvold and Marois 1995). However, susceptibility of wounds to *D. seriata* did not vary greatly between pruning times, similar to that reported in Italy by Serra *et al.* (2008). Contrasting trends were reported in France (Chapius *et al.* 1998), Michigan (Trese *et al.* 1982) and South Africa (van Niekerk *et al.* 2011), which may be due to environmental or climatic differences.

The mechanisms involved in wound repair have been discussed by a number of authors. Sun *et al*. (2006) reported that pruning caused rapid tylosis in grapevine shoots, which continued for several days, causing the wounds to seal rapidly. Biggs (1990) showed that suberin in wounds has an important role in disease resistance, and that at higher average temperatures, the rate of suberisation in wounded bark is greater than at lower temperatures. Munkvold and Marois (1995) reported that wound susceptibility to infection by *E. lata* declined significantly during the 28 days following pruning and that this decline was highly correlated with an increase in suberin and lignin deposition and with increased temperatures. A more rapid decrease in pathogen recovery (faster wound healing) coincided with lower temperatures and higher rainfall in Adelaide Hills (annual average temperature 14.4˚C and total rainfall 804 mm; data supplied by Adelaide Hills Wine Region) compared with McLaren Vale (15.8˚C and 601 mm, respectively; data supplied by McLaren Vale Grape, Wine and Tourism Association) and Wagga (15.8 ˚C and 561 mm; Bureau of Meteorology). Therefore, suberisation may not explain the differences between the current and previous studies.

Carter and Moller (1970) reported that apricot trees sheltered from winter rainfall were more susceptible to wound infection by *E. lata* than trees exposed to winter rainfall, and hypothesised that water-dispersed components of the tree surface microflora involved in the wound healing process may be denied a means of transport to the wound surfaces. According to the Californian study by Munkvold and Marois (1995), populations of microorganisms were greater in wounds made in early spring compared with those made in autumn or early winter. Henderson (2019) investigated the effect of rainfall on microflora present in grapevine pruning wounds in Australia. A lack of rainfall that occurred in June and July 2019 in the current study was associated with lower susceptibility of wounds to *E. lata*, so microorganism activity may not explain the resulting trends in wound susceptibility.

These results indicate that, in the Adelaide Hills, there may be an advantage in delaying pruning to later in the dormant season to minimise risk of infection by *E. lata*. In the case of *D. seriata*, there appears to be no real advantage in avoiding the early pruning time. However, over the course of three years of trials, only one incidence of natural infection of pruning wounds by *D. seriata* was recorded in the non-inoculated control wounds, and a spore trap located at the site detected very low levels of BD pathogen spores (Section 5.1), which indicated very little presence of this pathogen in this region. These results contrast with previous research (Ayres *et al.* 2016, Sosnowski *et al.* 2017, SAR1205) that showed little advantage in choosing one pruning time over another in the McLaren Vale and Wagga Wagga regions. The variability observed between different climatic regions highlights the importance of localised research to investigate wound susceptibility. Future research should focus on other climatic regions in order to ascertain the periods of greatest wound susceptibility.

Cryptovalsa ampelina is a trunk pathogen related to *E. lata*, which is found in grape growing regions throughout the world (Mostert *et al.* 2004, Luque *et al.* 2006, Trouillas *et al*. 2010b, Diaz *et al.* 2011). *C. ampelina* has been shown to be pathogenic to grapevine, similar to the pathogenicity of *E. lata*, although it does not produce the typical foliar symptoms of *E. lata* (Pitt *et al.* 2013). Over the past decade of trunk disease research, *C. ampelina* cultures have been increasingly observed growing from grapevine cane material on agar media (SAR 1205; Sosnowski *et al.* 2017). In the current research trials, *C. ampelina* was recovered from cane samples, often with higher frequency than *E. lata* and *D. seriata*, which were

artificially inoculated onto the pruning wounds, as well as in the NICs. In 2019-20, there was considerably less *C. ampelina* recovered from canes in the trial, which may be due to the lower rainfall recovered in that pruning season compared with the previous two years. Freshly cut canes were free of *C. ampelina* indicating that infection occurred post-pruning, and the prevalence of *C. ampelina* fruiting bodies on dead arms in the vineyard, suggested that this species may be playing a greater role in Eutypa dieback than previously thought.

Previous research (SAR 1001) reported that the fungicides tebuconazole and fluazinam, which are registered for use as pruning wound protectants for the control of *E. lata*, were shown to also be effective at inhibiting the growth of *C. ampelina in vitro* (Gramaje *et al.* 2012; Sosnowski *et al.* 2013a). However, the increased prevalence of *C. ampelina* in Australian vineyards requires further investigation. Firstly, it will be important to determine how widespread the species is across Australian growing regions. Its pathogenicity alone or together with *E. lata* and Botryosphaeriaceae species as part of a disease complex needs to be better understood. Further evaluation of wound protection treatments in the vineyard is also required, to confirm efficacy of currently recommended paints, fungicides and biocontrols.

A preliminary investigation of wound infection during spring shoot thinning has revealed that the two common wound types, pruning wounds caused during trimming of green shoots and socket wounds caused by the removal of green shoots at their base, were susceptible to infection by both *E. lata* and *D. seriata*. This result confirms previous reports regarding Botryosphaeria dieback pathogens (Úrbez-Torres 2011, Amponsah *et al.* 2012) and constitutes the first know report of spore infection of shoot thinning wounds by *E. lata*. With these results and the reports of spore detection of Eutypa and Botryosphaeria throughout spring, it will be important to determine the risk of infection in the vineyard following shoot thinning activities.

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8 PRUNING WOUND PROTECTION

8.1 Introduction

Spray application of fungicides to grapevine pruning wounds is an efficient and effective means to prevent infection by Eutypa and Botryosphaeria dieback pathogens (SAR 06/01, SAR 1001, Sosnowski *et al.* 2013b, Ayres *et al.* 2017b; Sosnowski and Mundy 2019). It was demonstrated that the level of disease control was correlated to pruning wound coverage, with a range of tractor driven sprayers providing similar control to that when fungicide was hand-applied with a paint brush. As most sprayers are designed to penetrate the vine canopy during the growing season, they require adjustment, including reducing pressure, re-directing and turning off nozzles and/or fans and using a high water output rate, in order to achieve adequate coverage of pruning wounds on dormant vines. This study aimed to evaluate the addition of adjuvants to fungicide treatments in order to increase wound coverage, and therefore improve protection against infection by GTD pathogens.

Only one organic product is currently registered for wound protection, so there is a need to seek more alternatives to synthetic fungicides, to support the growing demand for more sustainable vineyard practices. Detached cane assays (DCAs; SAR 1001, Ayres *et al*. 2011, 2017b) provide a method of pre-screening wound protectants in the controlled conditions of a greenhouse. The natural compounds vanillin and chitosan were reported to reduce infection by Botryosphaeria dieback and Esca pathogens in Spain (Cobos *et al.* 2015). *Aureobasidium pullulans* naturally occurs on grapevine pruning wounds and when artificially added to fresh pruning wounds was shown to reduce infection by *E. lata* (Munkvold and Marois 1993a, Babynec 2012). The biocontrol product, Botector (*A. pullulans*) has been registered for use in grapevines to control botrytis in Australia. This study evaluated chitosan, vanillin and Botector for protection of pruning wounds from infection by Eutypa and Botryosphaeria dieback pathogens.

Vinevax (*Trichoderma harzianum*) was the first product to be registered by the Australian Pesticides and Veterinary Medicines Authority (APVMA) as a wound protectant for Eutypa dieback control in 2004 (apvma.gov.au). However, when evaluated as part of a PhD thesis by John (2003), Trichoseal, the previous trade name of Vinevax, provided >80% control of Eutypa dieback compared with controls in two trials, <40% control in two trials, and no control in the fifth trial, which suggested variable efficacy, perhaps due to environmental conditions at the time of application and/or a time requirement for the antagonist to colonise the pruning wound. A more recent trial that evaluated wound protection against Botryosphaeria dieback pathogens reported <20% disease control (Pitt *et al.* 2012). Therefore, with increased reports on the impact of Botryosphaeria dieback in Australian vineyards (Taylor *et al.* 2005, Pitt *et al.* 2010, 2013a), it is important to evaluate Vinevax for wound protection against pathogens that cause Eutypa and Botryosphaeria dieback, and, in particular, to determine the time required for sufficient colonisation of the wounds by *T. harzianum* to provide protection against the pathogens.

8.2 Maximise wound coverage

8.2.1 Methods

A wound coverage trial was established to evaluate spray coverage and efficacy when two adjuvants: a sticker, Flextend (859 g/L a.i. di-1-p-menthene, applied at 50 mL/ 100 L; Agspec) and a wetter, Du-wett (500 g/L a.i. trisiloxane ethoxylate, applied at 50 mL/100 L; Nufarm Australia Ltd) were added to two registered fungicides: Gelseal (430 g/L a.i. tebuconazole, applied at 30 mL/100 L; Omnia Specialties Australia) and Emblem (500 g/L a.i. fluazinam, applied at 100 mL/100 L; Nufarm Australia Ltd).

In McLaren Vale SA, one-year-old canes (Shiraz, planted in 1996) were pruned to two buds using secateurs on 26 July 2017. All pruning wounds were covered with plastic bags (10 × 15 cm) secured with rubber bands (Figure 8.1a-b), except for the wounds that were about to be sprayed using an FMR Spray-Pro R Series recycle sprayer (water output rate 200 L/ha; Figure 8.1c). Following a given spray treatment, the treated canes were covered with plastic bags and the next set of canes exposed for the next spray treatment. This was repeated until all pruning wounds had received the appropriate spray treatment.



Figure 8.1. Wound coverage trial in McLaren Vale SA. (a-b) covering pruning wounds with plastic bags, (c) FMR Spray-Pro R Series recycle sprayer applying treatment, (d) pruning wound exposed to treatment containing fluorescent pigment viewed under UV light, (e-f) water sensitive papers in fixed positions on trellis post and on spurs and canes of vine following spray application.

For treatments, fungicides were applied alone, and with each adjuvant separately. Adjuvants were also applied alone, to check for efficacy. Each treatment was applied to 10 wounds on a vine, and the trial was designed as a randomised block with 10 replications, and included inoculated (IC) and non-inoculated controls (NIC).

Spray coverage was evaluated using fluorescent pigment (Yellow Pigment SC, Topline Paint Pty Ltd, Lonsdale, South Australia) added to the treatments. For each treatment, an additional five canes were pruned prior to spraying and covered with plastic bags as described above. Following the application of each treatment the five canes were removed from the vines and placed into paper bags to be returned to the laboratory for assessment. Digital images of the pruning wounds with fluorescent pigment were captured under UV light (Figure 8.1d) and assessed using Image J image analysis software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation - LOCI, University

of Wisconsin, USA), and a percent coverage of the pruning wounds was determined (Mangato *et al.* 2013). To compare with methods used in previous research (Ayres *et al*. 2017b, Sosnowski and Mundy 2019), four water sensitive papers (WSPs) were placed in fixed positions on a trellis post and an additional six WSPs were placed randomly attached to spurs and canes for each treatment (Figure 8.1e-f). The papers were removed from the vines after each treatment and placed in paper bags to be returned to the laboratory for analysis. The papers were scanned to a digital image and then assessed for percent coverage using Image J image analysis software.

On the following day, wounds were moistened by spraying with reverse osmosis water and then inoculated with 100 or 500 spores of *E. lata* suspended in 20 µL of sterile reverse osmosis (RO) water. *Eutypa lata* ascospores were obtained as described in Section 6.2. Two inoculum doses were used to maximise the possibility of having moderate recovery of 30–60% in the inoculated control, and each inoculation dose was assigned to five replications.

Treated canes were removed in May 2018 and returned to the laboratory for assessment. The bark was removed from each cane using a sharp knife, and the exposed wood was surface sterilised in 2.5% sodium hypochlorite (NaOCl) for 10 minutes and then washed twice in sterile RO water. Using flame-sterilised secateurs, canes were cut into segments ($3 \times 2 \times 2$ mm) taken from each side of the margin between live and dead wood tissue. Five wood segments were plated onto PDA amended with antibiotic (streptomycin sulfate 100 mg/L), with two plates used for each cane. Plates were incubated at 25°C under fluorescent light for 12 h each day for seven days and then assessed for presence or absence of mycelial growth of *E. lata*. Efficacy was based on the mean percent recovery (MPR) of *E. lata* from the treated canes. Data were subjected to analysis of variance and least significant difference (LSD) at the 5% level was used for all pair-wise comparisons.

The trial was repeated two more times, with pruning and treatment undertaken on 10 July 2018 and 3 July 2019, with canes then removed in May each following year.

8.2.2 Results

According to the fluorescent pigment, spray coverage on wounds for the different treatments ranged from 19 to 38% in 2017 (Figure 7.2). Emblem applied with the wetter added had the greatest wound coverage (38%), significantly more (p<0.05) than with any other treatment or the control (19%). The Gelseal treatment alone (28%) and with the sticker added (26%) had significantly greater coverage than the control (p<0.05). All other treatments had similar wound coverage to that of the control. In 2018, spray coverage on wounds for the different treatments ranged from 24 to 35%, and there was no difference in wound coverage between any treatments. In 2019, spray coverage on wounds ranged from 22 to 42%. Gelseal applied without an adjuvant had the greatest wound coverage (42%) significantly more than Gelseal with the wetter and Emblem with the wetter or without an adjuvant.

According to the WSPs, spray coverage on wounds for the different treatments ranged from 48 to 78% (2017), 60 to 75% (2018) and 28 to 64% (2019). Due to the limited number of WSPs used for each treatment, statistical analysis was not possible. Spray coverage measured by WSPs was often more than double that measured by the fluorescent pigment, and there was some correlation between the methods in 2017 (R^2=0.58), but not in 2018 (R^2=0.28) or 2019 (R^2=0.40).

In 2017/18, *E. lata* was recovered from 79% of IC canes and 5% of NIC canes, and recovery of *E. lata* from treated wounds ranged from 58 to 76% (Figure 7.3). Gelseal applied alone (58%) and Emblem with a wetter added (61%) had significantly reduced recovery (P<0.05) compared with the IC, but there were no other reductions in recovery when adjuvants were added to either fungicide. All other treatments had similar recovery to ICs. When applied alone, the sticker, wetter and fluorescent pigment had similar recovery of *E. lata* to the IC, ranging from 73-81% (data not shown).

In 2018/19, *E. lata* was recovered from 90% of IC canes and 6% of NIC canes and recovery of *E. lata* from treated wounds ranged from 58 to 77% (Figure 7.3). All treatments significantly reduced recovery (P<0.05) when compared with the IC, but there were no further reductions in recovery when adjuvants were added to either fungicide. When applied alone, the sticker, wetter and fluorescent pigment had similar recovery of *E. lata* to the IC, ranging from 87-90% (data not shown).

In 2019/20, *E. lata* was recovered from 64% of IC canes and 3% of NIC canes and recovery of *E. lata* from treated wounds ranged from 35 to 47% (Figure 7.3). All treatments significantly reduced recovery ($P<0.05$) when compared with the IC, but there were no further reductions in recovery when adjuvants were added to either fungicide. When applied alone, the sticker, wetter and fluorescent pigment had similar recovery of *E. lata* to the IC, ranging from 57 to 61% (data not shown).

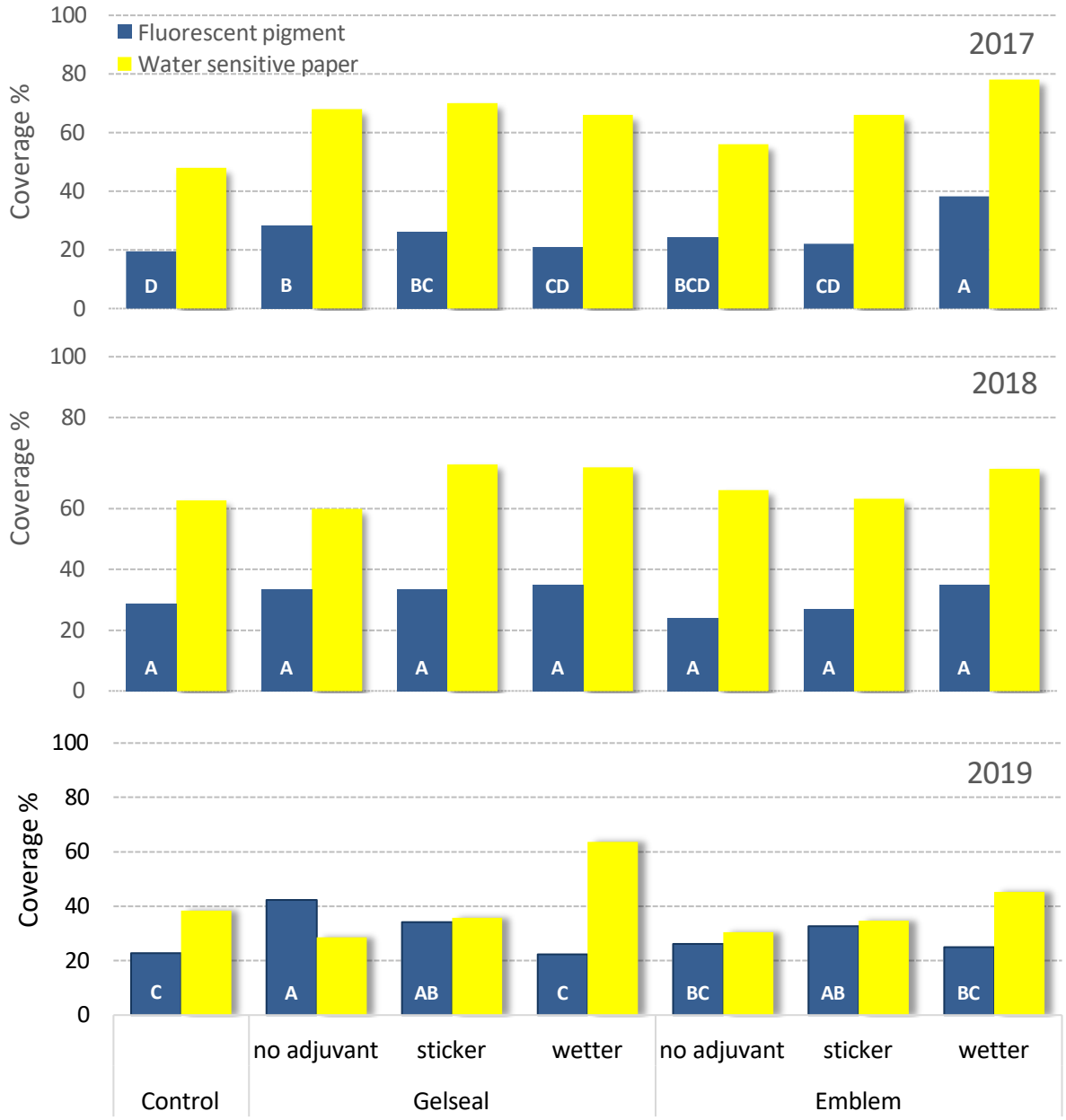


Figure 8.2. Spray coverage according to fluorescent pigment (blue) and water-sensitive papers (yellow) in spray trials conducted at McLaren Vale SA in 2017, 2018 and 2019. Control consists of fluorescent pigment in water. For the fluorescent pigment, columns with no letters in common are significantly different from each other ($P<0.05$).

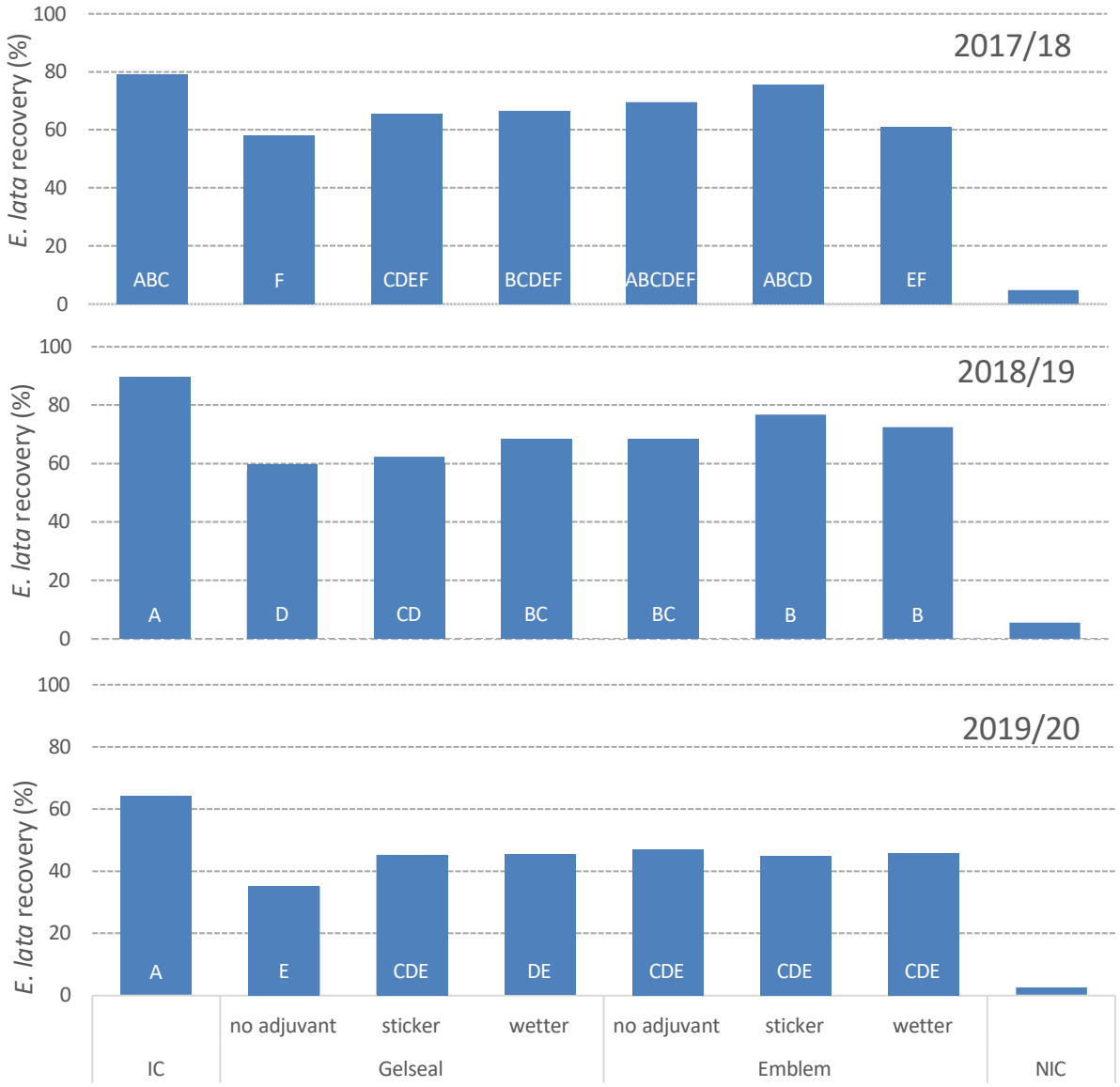


Figure 8.3. Recovery of *Eutypa lata* from canes treated with fungicides Gelseal (tebuconazole) or Emblem (fluazinam) with or without the addition of wetter (Du-wett) or sticker (Flextend), in a spray trial conducted at McLaren Vale, SA in 2017/18, 2018/19 and 2019/20. Columns with no letters in common are significantly different from each other (*P*<0.05).

8.3 Evaluate organic alternatives

8.3.1 Methods

8.3.1.1 Detached cane assays

A series of detached cane assay (DCA) experiments were established using methods described in Ayres *et al.* (2011). Canes (cv. Shiraz) were cut into 10 cm single-node sections with the top surface 1 cm above the node and placed into holes made in 20 mm thick polystyrene boards (500 x 350 mm), ensuring that the bottom of the canes extended at least 1 cm below the boards (Figure 7.2a). Boards were set up to hold 10 x 8 canes placed in rows across the boards. The boards with canes were floated on tap water in plastic tubs (Figure 7.2b) in a greenhouse maintained at approximately 25°C.

In DCAs 1-4, the treatments Botector ($5x10^9$ cfu *Aureobasidium pullulans* /g; Nufarm) at 1 g/L, Chitosan (Sigma Aldrich) at 12.5 and 25 mg/mL, Vanillin (Sigma Aldrich) at 5 and 10 mg/mL and, for comparison, Gelseal (430 g/L tebuconazole; Omnia Specialties Australia) at 0.3 mL/L water, were each applied by paintbrush to wounds. On the following day, wounds were inoculated with 100 spores of *E. lata* (ED; DCA 1-2) or *N. luteum* (BD; DCA 3-4) suspended in 20 µL SDW and applied using a pipette. Untreated controls were either inoculated with the spore suspension (IC) or not inoculated (NIC). *Eutypa lata* and *N. luteum* spores were obtained using the methods described in Sosnowski *et al*. (2017; SAR 1205). The experiments were set up as randomised block designs with five replications (boards), with a row of 10 canes for each treatment per board.

In DCAs 5-6, the treatment Vinevax ($5x10^8$ cfu *Trichoderma harzianum*) at 100 g/L and, for comparison, Gelseal at 0.3 mL/L water, were each applied by paintbrush to wounds. On the same day, and then 1, 3, 7, 14, 21 and 28 days later, wounds on top of canes were moistened by spraying with SDW immediately prior to inoculation with approximately 100 spores of *E. lata* (ED; DCA 5) or *N. luteum* (BD; DCA 6) suspended in 20 µL SDW and applied using a pipette. Untreated controls were either inoculated with the spore suspension (IC) or not inoculated (NIC). The *E. lata* DCA was set up as randomised block design with four replications, and a row of five canes for each treatment and time combination. The *N. luteum* DCA was set up as randomised block design with 10 replications, and three canes for each treatment and time combination.

For all DCAs, four weeks after the last inoculation, canes were removed from boards and processed as described in Sosnowski *et al*. (2017; SAR 1205). The bark was removed from each cane using a sharp knife. The exposed wood was surface sterilised in 2.5% sodium hypochlorite (NaOCl) for 10 minutes (*E. lata*) or 3 minutes (*N. luteum*) and then washed twice in SDW. For *E. lata* experiments, using flame-sterilised secateurs, canes were cut into small segments that spanned the interface between normal and stained wood tissue. Five randomly selected wood segments were plated in duplicate onto PDA amended with antibiotic (streptomycin sulfate 100 mg/L). For *N. luteum* experiments, canes were cut into four segments at the margin and placed onto one plate. Samples were incubated at 25°C under fluorescent light for 12 h each day (SA) or total darkness (NSW) for seven days and then assessed for presence or absence of mycelial growth of *E. lata* or *N. luteum*.

Data were subjected to analysis of variance (Statistix for Windows v 8, Tallahassee, FL) and used the least significant difference (LSD) all-pair-wise comparisons test at the 5% level.

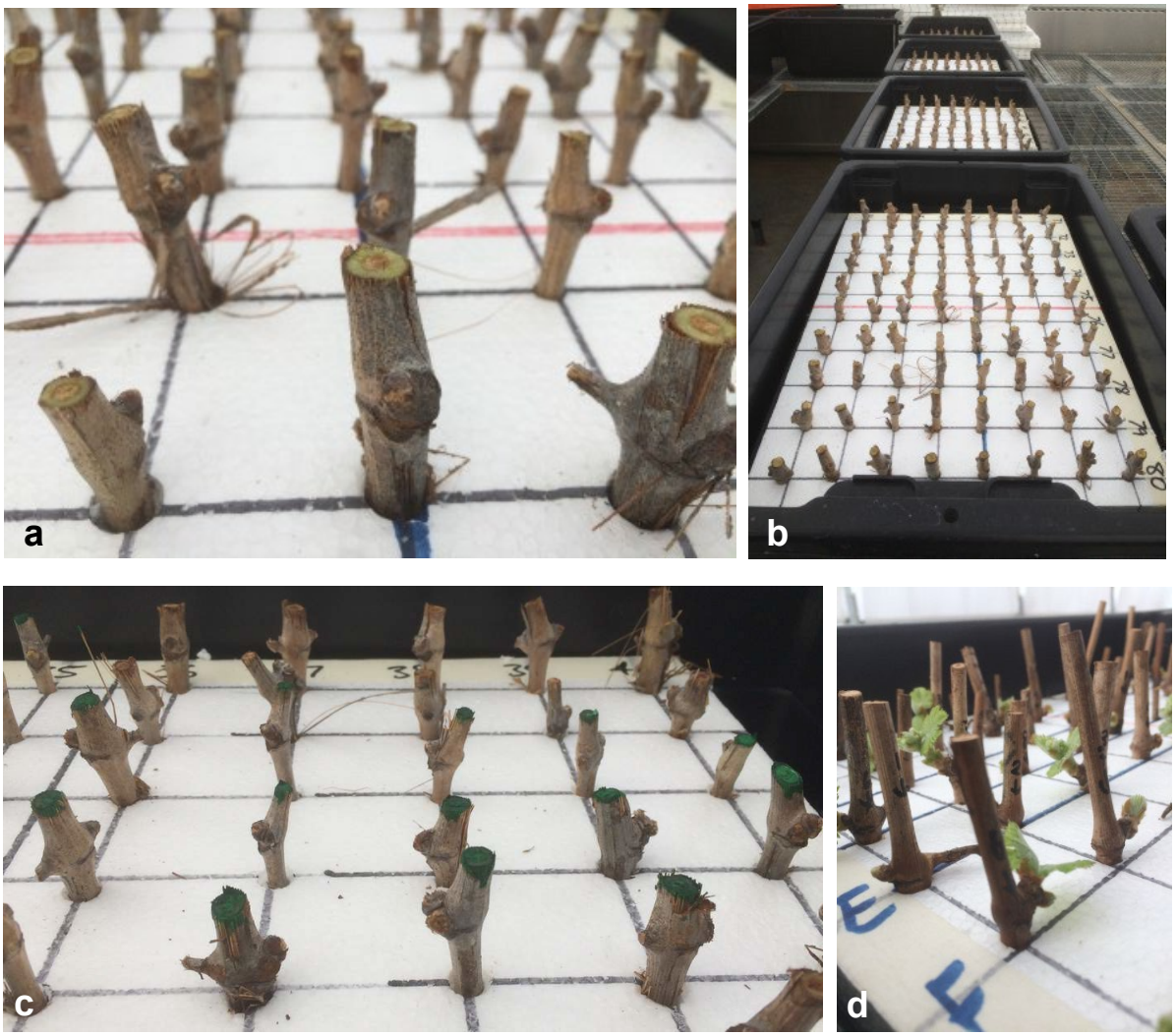


Figure 8.4. Detached cane assays; (a) single node canes inserted into holes in polystyrene boards, (b) boards floating in tubs of water on greenhouse bench (c) Vinevax (green) treatment applied to wounds and (d) buds bursting on canes.

8.3.1.2 Field trials

Vinevax

A vineyard trial was established in 2017 and repeated in 2018 on cv. Shiraz vines planted in 1997 near Hahndorf, in the Adelaide Hills, South Australia to evaluate the efficacy of applying Vinevax ($5x10^8$ cfu/g *Trichoderma harzianum*) and Gelseal (430 g/L tebuconazole) to wounds on the day of pruning, to control infection by spores of *E. lata* and *D. seriata* at different times following pruning.

One-year-old canes were pruned to two buds, using secateurs, on 18 July 2017 and 16 July 2018. Using a paintbrush, wounds were treated with 100 g/L Vinevax or 0.3 mL/L Gelseal on the day of pruning. Then, following treatment, on the day of pruning (Day 0), as well as 1, 3, 7, 14, 21 and 28 days after pruning and treatment, wounds were moistened by spraying with sterile distilled water (SDW) immediately prior to inoculation with approximately 200 spores of *E. lata* or *D. seriata* suspended in 20 µL SDW and applied using a pipette. *E. lata* and *D. seriata* spores were obtained using the methods described in Sosnowski *et al.* (2017; SAR 1205). Controls, positive (inoculated, IC) and negative (non-inoculated; NIC) were treated with SDW. Each treatment was allocated to a vine with 10 pruned canes. The trial was set up as randomised block design with four replications.

Canes were harvested from vines in May 2018 and 2019 and returned to the laboratory for assessment. Bark was removed from each cane using a sharp knife, and the exposed wood was surface sterilised in 2.5% sodium hypochlorite (NaOCl) for 10 minutes and then washed twice in SDW. Using sterilised

secateurs, canes were cut into segments (3 × 2 × 2 mm) taken from each side of the margin between live and dead wood tissue. Five wood segments were plated in duplicate onto PDA amended with antibiotic (streptomycin sulfate 100 mg/L). Plates were incubated at 25°C under fluorescent light for 12 h each day for seven days and then assessed for presence or absence of mycelial growth of *E. lata* or *D. seriata*. In addition, presence or absence of *Trichoderma* was also recorded for each cane.

Data were analysed using a binomial generalised linear model (GLM) with a logit link (Genstat 19th Ed; VSN International Ltd, Hemel Hempstead, UK). A Wald test was used to determine significant differences between treatments, and a Bonferroni test was used to compare between treatments, separately at each inoculation time. Efficacy was presented as the mean percentage recovery (MPR) of *E. lata* or *D. seriata* from the treated canes by isolation on PDA.

Botector

A vineyard trial was established in 2019 and repeated in 2020 on cv. Shiraz vines planted in 1997 near Hahndorf, in the Adelaide Hills, South Australia to evaluate the efficacy of applying Botector ($5x10^9$ cfu/g *Aureobasidium pullulans*) and Gelseal (430 g/L tebuconazole) to wounds on the day of pruning to control infection by spores of *E. lata* and *D. seriata* at different times following pruning.

One-year-old canes were pruned to two buds, using secateurs, on 18 June 2019 and 6 July 2020. Using a paintbrush, wounds were treated with 1 g/L Botector or 0.3 mL/L Gelseal on the day of pruning. Then, following treatment, on days 1, 3, 7 and 14 days after pruning and treatment, wounds were moistened by spraying with sterile distilled water (SDW) immediately prior to inoculation with approximately 200 spores of *E. lata* or *D. seriata* suspended in 20 µL SDW and applied using a pipette. *E. lata* and *D. seriata* spores were obtained using the methods described in Sosnowski *et al.* (2017; SAR 1205). Controls, positive (inoculated, IC) and negative (non-inoculated; NIC) were treated with SDW. Each treatment was allocated to a vine with 10 pruned canes. The trial was set up as randomised block design with four replications.

Canes were harvested from vines in May 2020 and 2021 and returned to the laboratory for assessment. Bark was removed from each cane using a sharp knife, and the exposed wood was surface sterilised in 2.5% sodium hypochlorite (NaOCl) for 10 minutes and then washed twice in SDW. Using sterilised secateurs, canes were cut into segments (3 × 2 × 2 mm) taken from each side of the margin between live and dead wood tissue. Five wood segments were plated in duplicate onto PDA amended with antibiotic (streptomycin sulfate 100 mg/L). Plates were incubated at 25°C under fluorescent light for 12 h each day for seven days and then assessed for presence or absence of mycelial growth of *E. lata* or *D. seriata*. In addition, presence or absence of *A. pullulans* cultures was also recorded for each cane.

Data were analysed using a binomial generalised linear model (GLM) with a logit link (Genstat 19th Ed; VSN International Ltd, Hemel Hempstead, UK). A Wald test was used to determine significant differences between treatments, and a Bonferroni test was used to compare between treatments, separately at each inoculation time. Efficacy was presented as the mean percentage recovery (MPR) of *E. lata* or *D. seriata* from the treated canes by isolation on PDA.

8.3.2 Results

8.3.2.1 Detached cane assays

In DCAs 1 and 2 for ED, *E. lata* was recovered from 46 and 49% of inoculated control canes but was not recovered from non-inoculated canes (Figure 7.3). When applied to wounds, Gelseal, Botector and chitosan significantly reduced (P<0.05) recovery of *E. lata* to below 26% (DCA 1) and below 16% (DCA 2), compared with inoculated controls. Vanillin only significantly reduced (P<0.05) recovery of *E. lata* to 30% when applied to wounds at 5 mg/mL in DCA 1.

In DCAs 3 and 4 for BD, *N. luteum* was recovered from 98 and 34% of inoculated control canes and there was no recovery from non-inoculated canes (Figure 7.4). When applied to pruning wounds, Gelseal significantly reduced (P<0.05) *N. luteum* recovery to 32 and 2%, in DCAs 1 and 2, respectively, compared with inoculated controls. Botector reduced recovery of *N. luteum*, but only significantly (P<0.05) to 68% compared with the inoculated control in DCA 1. Chitosan and vanillin did not reduce recovery of *N. luteum* in either DCA.

In DCA 5 for ED, *E. lata* was recovered from 95% of canes inoculated at day 0, and gradually decreased to 23% for those inoculated at day 14, after which *E. lata* was not recovered at all, and no *E. lata* was recovered from non-inoculated canes (Figure 7.5). Gelseal application to wounds significantly reduced (P<0.05) recovery to 0-38%, compared with the ICs, for all of the inoculation times. *E. lata* was not recovered from any canes treated with Vinevax. It was noted that all PDA plates containing wood chips taken from Vinevax treated canes, were rapidly swamped with mycelium of *Trichoderma* sp.

In DCA 6 for BD, *N. luteum* was recovered from 100% of canes inoculated at day 0, and erratically ranged between 13 and 100% for all inoculation times to day 28. No *N. luteum* was recovered from non-inoculated canes (Figure 7.5). Gelseal application to wounds significantly reduced (P<0.05) recovery to between 0 and 23%, compared with the ICs, for all of the inoculation times. *N. luteum* was recovered from 3% of canes treated with Vinevax and inoculated on day 0, but not for any other inoculation time. As with DCA 5, it was noted that all PDA plates containing wood chips taken from Vinevax treated canes, were rapidly swamped with mycelium of *Trichoderma* sp.

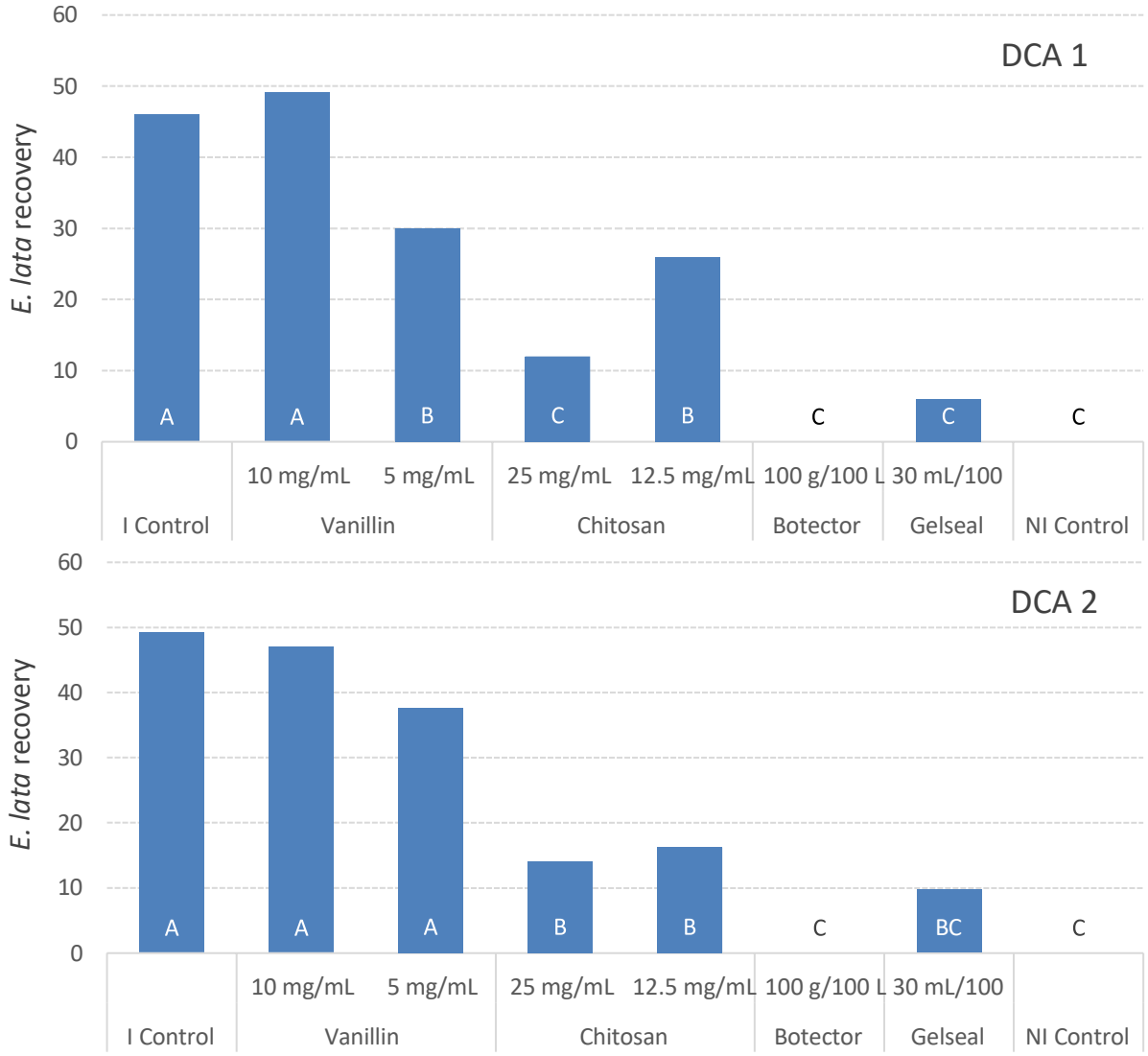


Figure 8.5. Incidence of *Eutypa lata* recovery from Shiraz canes in a detached cane assay. Treatments were applied to wounds on the day of pruning and then inoculated with 100 spores on the following day. I Control = inoculated control, NI Control = non-inoculated control. Columns with no letters in common are significantly different from each other (P<0.05).

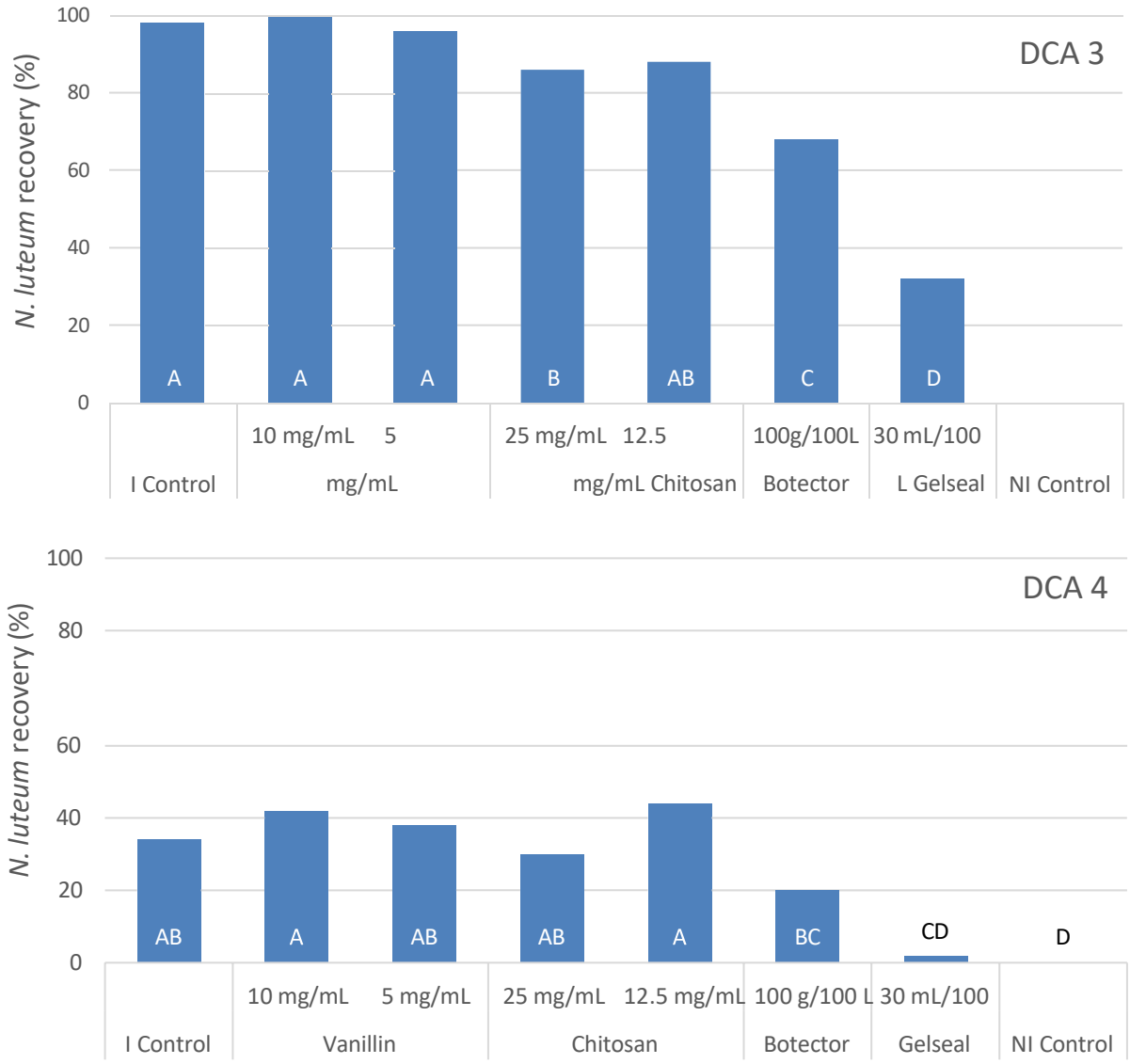


Figure 8.6. Incidence of *Neofusicoccum luteum* recovery from Shiraz canes in a detached cane assay. Treatments applied to wounds on the day of pruning and then inoculated with 100 spores on the following day. I Control = inoculated control, NI Control = non-inoculated control. Columns with no letters in common are significantly different from each other ($P<0.05$).

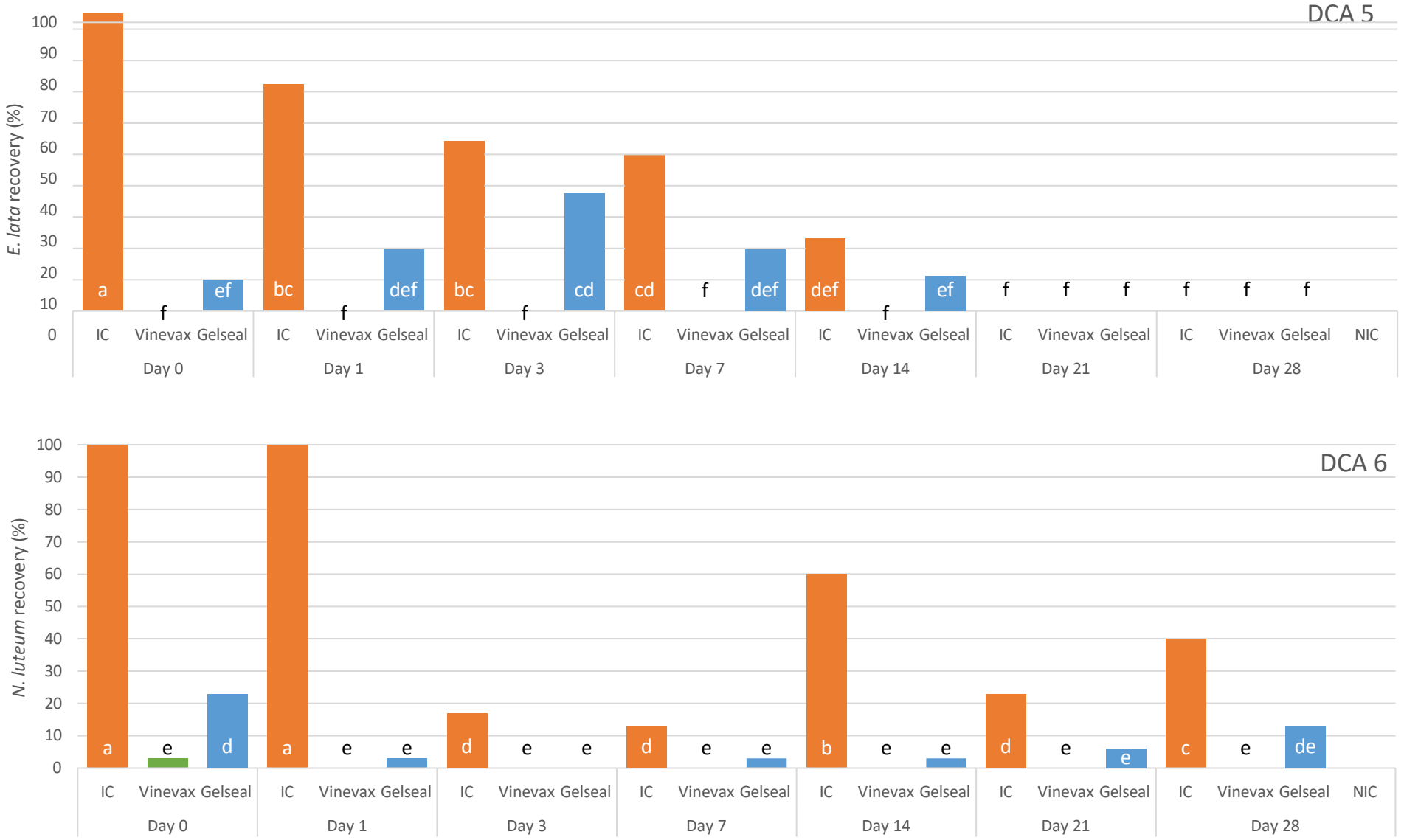


Figure 8.7. Incidence of *Eutypa lata* recovery from Shiraz canes in a detached cane assay. Treatments applied to wounds on the day of pruning and then inoculated with 100 spores on the same day (Day 0), and then 1, 3, 7, 14, 21 and 28 days after pruning. IC = inoculated control, NIC = non-inoculated control. Columns with no letters in common are significantly different from each other (*P*<0.05).

8.3.2.2 Field trials

Vinevax

Mean recovery of *Trichoderma* ranged from 49-64% of canes across pathogen inoculation treatment times (Figure 8.8).

As there was no significant difference (*P*>0.05) between the two trials, data were combined for statistical analysis. *E. lata* was recovered from 48 and 11% of IC (Day 0) and NIC canes, respectively, and *D. seriata* was recovered from 52 and 2% of IC (Day 0) and NIC canes, respectively. (Figure 8.9).

Mean recovery of *E. lata* and *D. seriata* was significantly (*P*<0.05) reduced with the application of Gelseal when inoculated up to three days post-pruning and treatment compared with ICs (Figure 8.9). When inoculated 7 to 28 days post-pruning, Gelseal reduced recovery of *E. lata* and *D. seriata*, but differences were not significant, and generally similar to that of NICs.

Compared with ICs, mean recovery of *E. lata* reduced slightly with the application of Vinevax, except when inoculated at seven days post-pruning and treatment, but reductions were not significant (*P*>0.05; Figure 8.9). Mean recovery of *D. seriata* was also reduced slightly compared to ICs with the application of Vinevax, but only significantly (P<0.05) when inoculated at three days post-pruning and treatment.

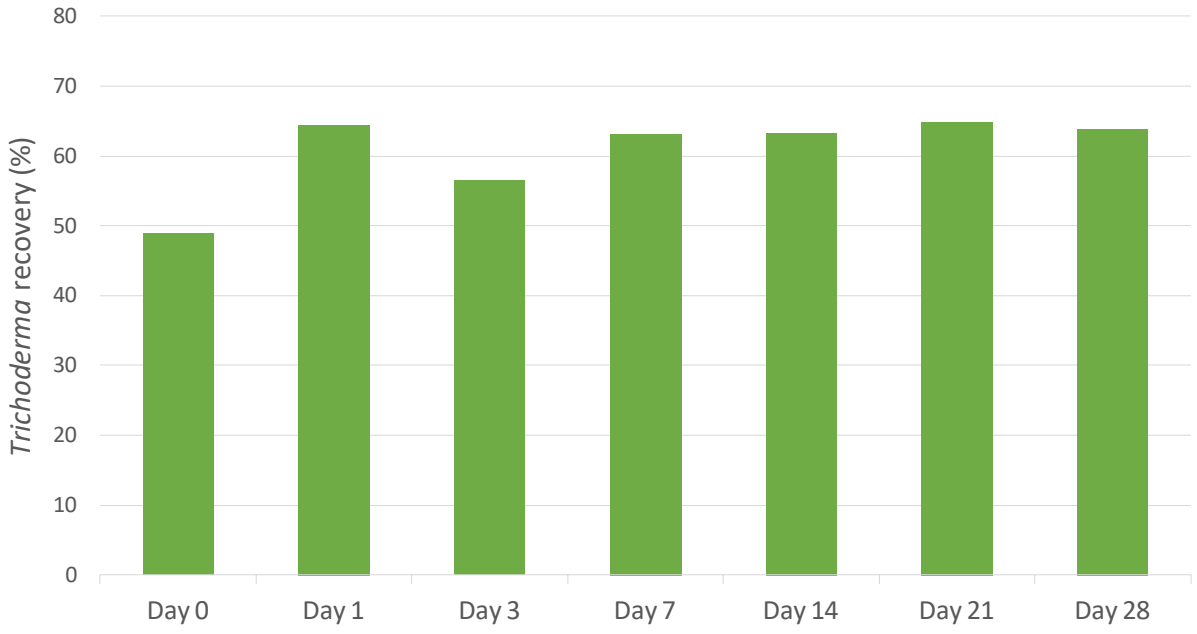


Figure 8.8. Mean percent recovery of *Trichoderma* from canes pruned and treated with 100 g/L Vinevax (5x10^8 cfu *Trichoderma harzianum*) on the same day, prior to inoculation with 200 spores of *Eutypa lata* (Eutypa dieback) or *Diplodia seriata* (Botryosphaeria dieback) on the same day (0), and 1, 3, 7, 14, 21 and 28 days after pruning, in a vineyard near Hahndorf in the Adelaide Hills, South Australia. Data for the repeated trial over two years and for both pathogens were combined.

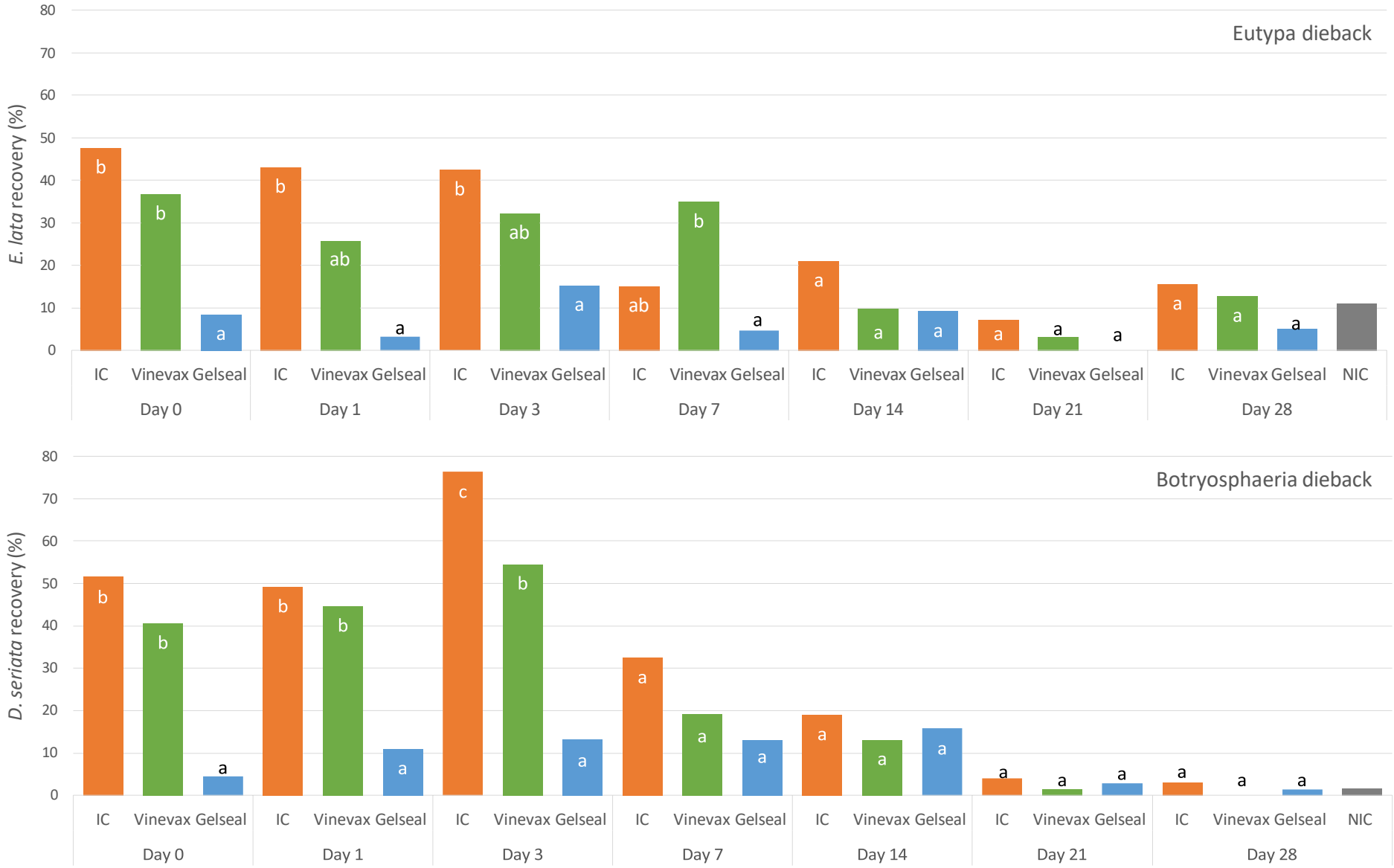


Figure 8.9. Mean percent recovery of *Eutypa lata* (Eutypa dieback; top) and *Diplodia seriata* (Botryosphaeria dieback; bottom) from canes pruned and treated with 100 g/L Vinevax (5x10^8 cfu *Trichoderma harzianum*) or 0.3 mL/L Gelseal (430 g a.i./L tebuconazole) and inoculated with 200 *E. lata* or *D. seriata* spores on the same day (0) and at 1, 3, 7, 14, 21 and 28 days after pruning, in a vineyard near Hahndorf in the Adelaide Hills, South Australia. Data for the repeated trial over two years were combined. IC = inoculated control, NIC = non-inoculated control. Columns with no letters in common are significantly different from one another according to Bonferroni's test.

Botector

In 2019/20, the mean recovery of *A. pullulans* ranged from 11-29% of canes treated with Botector ($5x10^9$ cfu/g *A. pullulans*), and from 0-5% of canes not treated with Botector, across pathogen inoculation treatment times (Figure 8.10). In 2020/21, *A. pullulans* was only isolated from three canes across the two trials, two were treated with Botector and a third was an NIC (data not shown).

As data were similar between the two trials, they were combined for statistical analysis. *E. lata* was recovered from 54 and 1% of IC (Day 1) and NIC canes, respectively, and *D. seriata* was recovered from 77 and 3% of IC (Day 1) and NIC canes, respectively. (Figure 8.11).

Mean recovery of *E. lata* was significantly ($P<0.05$) reduced with the application of Gelseal when inoculated up to seven days post-pruning and treatment, compared with ICs (Figure 8.11). When inoculated 14 days post-pruning, recovery of *E. lata* from Gelseal treated canes was similar to that of NICs. Mean recovery of *D. seriata* was significantly ($P<0.05$) reduced with the application of Gelseal when inoculated up to 14 days post-pruning and treatment (with the exception of seven days where reduction was not significant), compared with ICs (Figure 8.11).

Compared with ICs, mean recovery of *E. lata* and *D. seriata* was not significantly less ($P>0.05$) than IC controls (Figure 8.11).

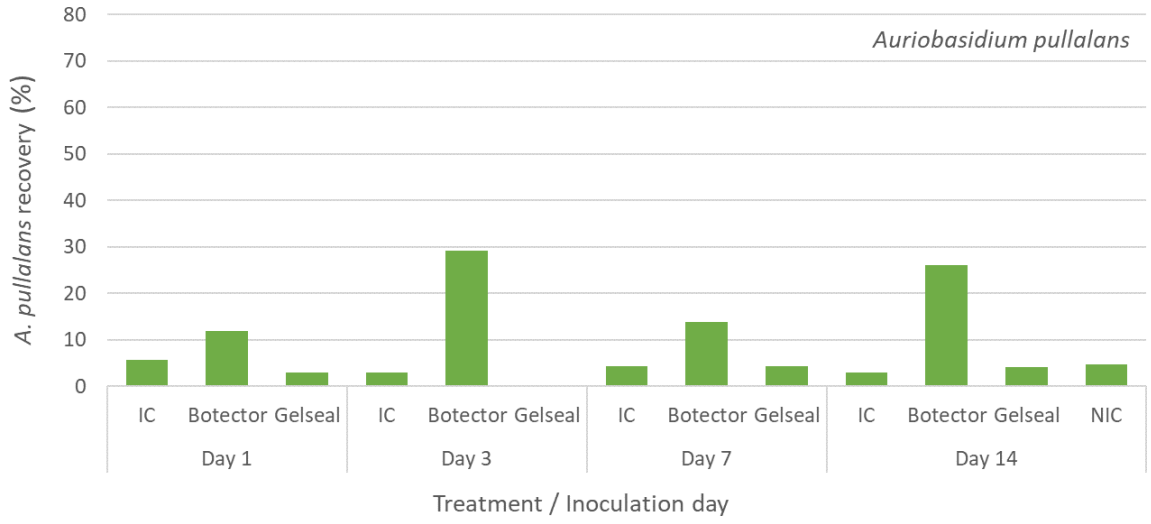


Figure 8.10. Mean percent recovery of *Aureobasidium pullulans* from canes pruned and treated with 1 g/L Botector ($5x10^9$ cfu/g *A. pullulans*) on the same day, prior to inoculation with 200 spores of *Eutypa lata* (Eutypa dieback) or *Diplodia seriata* (Botryosphaeria dieback) on 1, 3, 7 and 14 days after pruning in June 2019, in a vineyard near Hahndorf in the Adelaide Hills, South Australia.

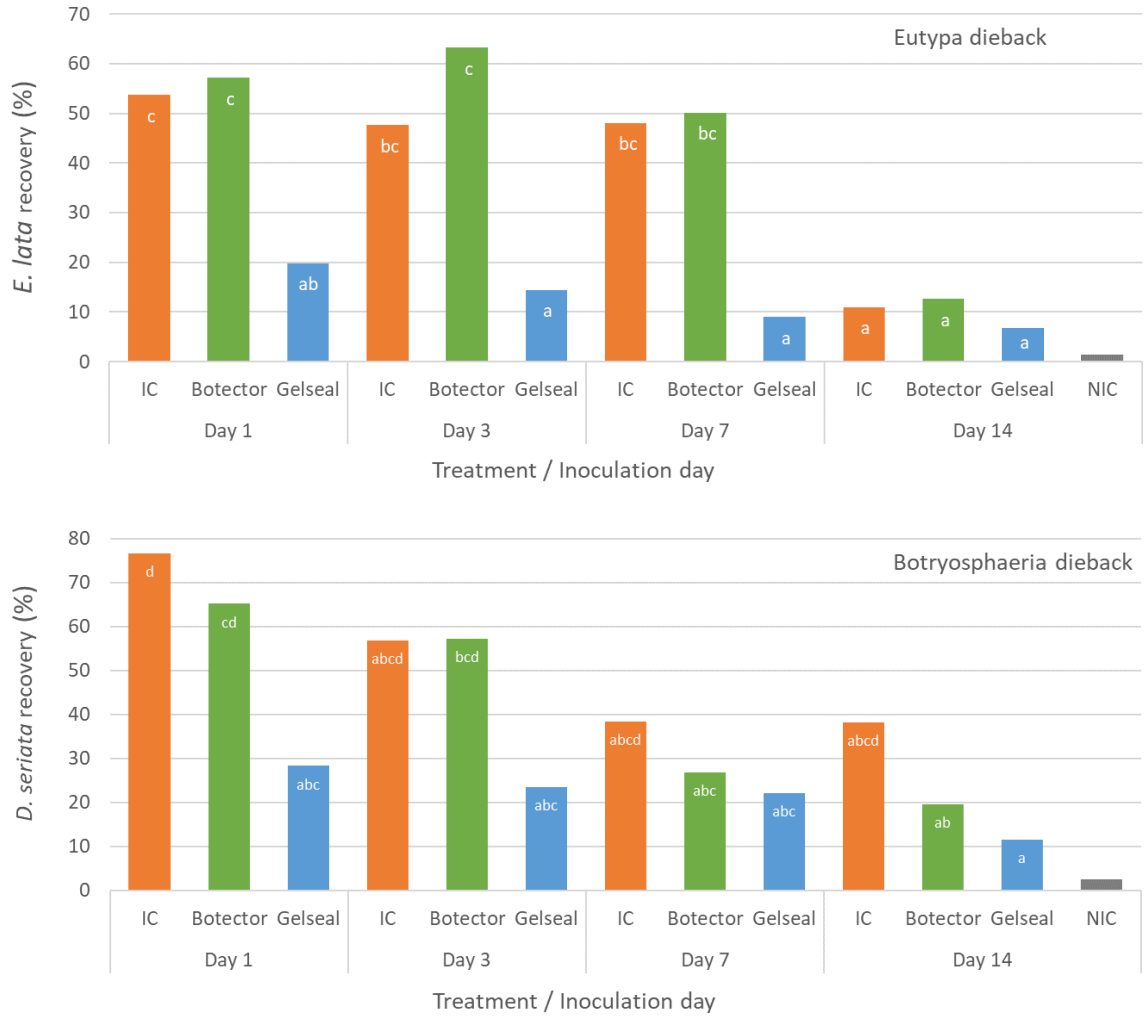


Figure 8.11. Mean percent recovery of *Eutypa lata* (Eutypa dieback; top) and *Diplodia seriata* (Botryosphaeria dieback; bottom) from canes pruned and treated with 1 g/L Botector ($5x10^9$ cfu/g *Aureobasidium pullulans*) or 0.3 mL/L Gelseal (430 g a.i./L tebuconazole) and inoculated with 200 *E. lata* or *D. seriata* spores on 1, 3, 7 and 14 days after pruning, in a vineyard near Hahndorf in the Adelaide Hills, South Australia. Data for the repeated trial over 2 years were combined. IC = inoculated control, NIC = non-inoculated control. Columns with no letters in common are significantly different from one another according to Bonferroni's test.

8.4 Discussion

8.4.1 Maximise wound coverage

Results from the three years of the wound spray coverage trial indicate that there is no benefit from adding adjuvants to improve coverage and efficacy of fungicides.

In previous research (SAR 1001; Sosnowski *et al.* 2013a), the fungicides Folicur (tebuconazole) and Cabrio (pyraclostrobin) were applied, with and without adjuvants Pentrabark and Du-wett, added respectively, and sprayed onto wounds with a hand-held sprayer until runoff. For each fungicide, there was no increased disease control by adding the adjuvant, however it was concluded that spraying to runoff provides no possibility of improving coverage and efficacy. As a result, for the current study, a relatively low water rate (200 L/ha) was used to provide sub-optimal coverage of pruning wounds, which provided an opportunity for the addition of spray adjuvants to improve wound coverage. The optimal recommended water rate for spraying wounds is 600 L/ha or more (Ayres *et al.* 2017b).

Digital images were captured of both the fluorescent pigment on wounds and from WSPs, and spray coverage was analysed using Image J software. Overall, there was little effect of treatment on coverage, indicating that the adjuvants tested did not increase wound coverage. However, when compared directly, WSPs indicated more than twice the coverage compared to the fluorescent pigment deposited on pruning wounds from the same treatment, in the first two years of trials, although differences were not as pronounced in the third year of trials. This difference may be due to the difference in surface of a WSP compared with wound tissue. WSPs are designed to mimic a leaf, which has a waxy layer and allows water droplets to run across the surface. The surface of a wound is more 'sponge' like, tending to allow water droplets to land and soak into the tissue rapidly. Although relative coverage can still be more easily estimated using WSPs, it is important to recognise that actual coverage of the wounds may be less than indicated. The development of a protocol for using fluorescent pigment to monitor spray coverage on pruning wounds may be a useful tool for future research and could be used by operators to adjust and calibrate sprayers for optimal spray coverage.

Treated canes were removed 11 months after pruning, treatment and inoculation, and assessed for pathogen recovery in the laboratory. Overall, there was little difference in recovery between treatments, indicating that the adjuvants tested did not improve efficacy of fungicides. Also of note is the relatively poor disease control that was achieved with the application of the two registered fungicides. The reason for this is the low water rate that was used (200 L/ha), reiterating the importance of applying the recommended minimum of 600 L/ha to achieve sufficient coverage (Ayres *et al.* 2017b).

8.4.2 Evaluate organic alternatives

Alternative products were evaluated for protection of pruning wounds against infection by ED and BD pathogens in a series of DCAs. For ED, Botector and chitosan both showed promise, considerably reducing recovery of *E. lata.* For BD, *N. luteum* recovery was reduced, albeit to a lesser extent, when treated with Botector, whereas chitosan appeared to afford little protection.

In the DCAs that evaluated the application of Vinevax (*Trichoderma harzianum*) followed by inoculations from 0-28 days, no *E. lata* was recovered from any wounds and there was only minimal recovery of *N. luteum*. It was noted that the PDA plates were rapidly overrun by *Trichoderma* species, which may have impeded the growth of the slower growing pathogens on the agar, even if they were present in the canes.

To further evaluate the timing of application of *Trichoderma*, a vineyard trial was established to evaluate Vinevax applications followed by the same inoculation timing from 0-28 days. *E. lata* recovery was slightly reduced compared to inoculated controls, but not significantly, whereas Gelseal significantly reduced *E. lata* recovery when inoculated up to seven days after pruning, with only small, insignificant reductions for inoculations 14-28 days after pruning. *D. seriata* recovery was only significantly reduced when treated with *Trichoderma* and inoculated three days after pruning, with only insignificant reductions at all other inoculation times.

The difference in results between the DCAs and field trials may be due to the incubation time. With DCAs, canes are harvested and assessed four weeks after the final inoculation, whereas the field trials are harvested and assessed about 11 months after the trial was established. This extra time may allow the pathogen, if it successfully infects the wound, to establish and colonise the cane more extensively, so it can outgrow the *Trichoderma* sp. on agar. However, these results with Vinevax suggest that it is not a reliable wound protectant against ED and BD pathogens. There have been conflicting reports from other countries showing that *Trichoderma* spp. were ineffective at protecting pruning wounds from infection by *D. seriata* in Spanish vineyards (Martínez-Diz *et al.* 2021) but more promising results were reported by J. Úrbez-Torres (Pers. comm. 2022) with disease control by *Trichoderma* spp. similar to that of Gelseal (tebuconazole) in Canadian vineyards. For the latter report, local acquisition of *Trichoderma* isolates was considered to be important for their efficacy.

Of note in the current study is the high incidence of *Trichoderma* re-isolated from Vinevax treated canes 11 months after application, suggesting successful and sustained colonisation. It is possible that it may take more than several weeks for *Trichoderma* to sufficiently colonise and become competitive or antagonistic to pathogen spores. Further longer-term investigation is necessary on application of higher *Trichoderma* spore concentrations on wounds to speed up colonisation, evaluating other *Trichoderma* products and species, and screening of local isolates from Australian wine regions.

Botector (*Aureobasidium pullulans*), which is already registered for control of Botrytis bunch rot (*Botrytis cinerea*), showed promise in the DCA experiments, significantly reducing recovery of *E. lata* and, to a lesser extent, *D. seriata*. However, when evaluated in the vineyard, there was no effect on wound infection by either pathogen. Unlike *Trichoderma*, *A. pullulans* was re-isolated at low incidence 11 months after application, suggesting either poor or temporary colonisation. Again, further investigation is required to evaluate the efficacy of *A. pullulans* when applied at higher concentrations on wounds following pruning.

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9 REMEDIAL SURGERY

9.1 Introduction

Remedial surgery has long been used for control of dieback diseases (Carter 1994; Creaser and Wicks, 2004; Leavitt 1990), and was demonstrated to control Eutypa dieback (ED) in grapevines (Sosnowski *et al.* 2011; SAR 06/01). Current recommendations are to cut out all vine wood visibly affected by ED and an additional 20 cm of non-symptomatic wood (Sosnowski 2021). The infected wood should be burnt, buried or composted. Watershoot(s) produced from the vine stump are trained to replace the missing vine or cordon(s).

Several trials were initiated in 2013 and 2014 to evaluate the efficacy of remedial surgery for control of Botryosphaeria dieback (BD; SAR 1205; Sosnowski *et al.* 2017). Short-term results revealed the difficulty of inducing watershoots from the scion of trunks of vines on rootstocks, especially when cuts were made close to the graft union. In order to ascertain medium-term success of remedial surgery to control BD, it was proposed that the existing field trials continue to be monitored and a new trial be established on grafted vines.

Emergence of watershoots following remedial surgery can be sporadic, with reports of as few as 42% of Shiraz trunks around the age of 30 years producing watershoots (Sosnowski *et al.* 2011; SAR 06/01). Anecdotally, a number of methods have been reported to stimulate watershoot production, so a trial was established to evaluate methods of inducing watershoots following remedial surgery.

9.2 Methods

9.2.1 Remedial surgery trials

A remedial surgery trial was established in the King Valley, Vic in October 2017. The vineyard comprised 28-year-old Shiraz grafted on Schwarzmann rootstock. Prior to remedial surgery at each site, the severity of dieback was visually assessed for each grapevine, from 0% (healthy) to 100% (dead) (Figure 9.1a). Following assessment, the trunks were cut using a chainsaw (Figure 9.1b) either high (just below crown), mid (mid-point between ground and crown) or low (20 cm above the graft union; Figure 9.1c), cordons and trunks removed, and the cross-section of each trunk was assessed for percentage of necrotic staining in the form of a wedge and/or central staining around the pith (Figure 9.2a-c) while uncut vines were included as negative controls. All cut trunks were sealed with Greenseal™ Pruning Wound Dressing (10g/L tebuconazole) (Figure 9.2d-e). Sub-samples of symptomatic wood were assessed for the presence of trunk disease fungi by isolating onto artificial media. The production of watershoots was recorded during the following growing seasons and the highest shoot was trained to form a new trunk.



Figure 9.1. a) Shiraz vine with dieback (arrow) in King Valley, Vic vineyard prior to remedial surgery, (b) trunk being cut with a chain saw below the crown and (c) low-cut vines after remedial surgery.



Figure 9.2. (a-c) Trunk cross-sections of symptomatic vines showing necrotic staining in the form of a wedge and/or central staining and (d-e) pruning wound dressing applied following remedial surgery.

The existing remedial surgery trial in Hilltops, NSW was established in November 2014. The vineyard comprised 24-year-old own-rooted Shiraz vines. Prior to remedial surgery, the severity of dieback was visually assessed for each grapevine as described in SAR 1205 (Sosnowski *et al.* 2017). Following assessment, the trunks were cut with a chainsaw either high, mid or low and the cross-section of each trunk was assessed for percentage of necrotic staining in the form of a wedge and/or central staining around the pith as described in SAR 1205 (Sosnowski *et al.* 2017) while uncut vines were included as negative controls.

9.2.2 Watershoot induction

A watershoot induction trial was established in 2017 in a Cabernet Sauvignon vineyard planted in 1995 near Auburn in the Clare Valley, South Australia. Prior to a mid-winter remedial treatment on 21 June, vines were visually assessed for overall severity of trunk disease symptoms (ED and BD), which included presence of at least two dead spurs, cordon dieback and trunk cankers (Figure 9.3a). Ratings were on a percentage scale (0% - non-symptomatic, 100% - dead; see Section 10.2.1).

Treatments aimed at inducing watershoot production were undertaken in an area of the trunks pre-designated by painting a white circle. A number of the treatments were chosen based on anecdotal reports from grapegrowers of their success in stimulating watershoot production, and included hitting with a hammer, making an X-shaped cut with a tomahawk and rubbing bark with a wire brush (Figure 9.3b-d).

Cross-sectional cuts through trunks were then made with a chainsaw below the cordon and above the irrigation wire (approximately 30 cm from ground), and the severity of disease staining was assessed in the cross-sections (Figure 9.3e-i). Wounds were painted with Greenseal™ immediately following surgery (Figure 9.3j). The plant growth regulator treatment Dormex® was then applied to designated trunks with a backpack sprayer (Figure 9.3k). Spring remedial treatments were undertaken on 27 September as described for the winter treatment.

To determine the causal trunk pathogens, sections of excised trunk (approx. 5 cm long) with staining were returned to the laboratory for diagnostic assessment. The bark was removed from each sample using a sharp knife, and wood was surface sterilised in 2.5% sodium hypochlorite (NaOCl) for 10 minutes and then washed twice in SDW. Holes were made with a flame-sterilised drill bit so that shavings retrieved contained both stained and clean wood tissue. Five wood shaving segments (2 x 2 mm) were placed onto plastic Petri dishes filled with potato dextrose agar (PDA) amended with antibiotic (streptomycin sulfate 100 mg/L). Following incubation for seven days, cultures resembling those of known trunk pathogens were sub-cultured onto fresh PDA. Cultures were then observed for 2-3 weeks and indentified using morphological characteristics.

All grafting treatments were conducted by T-budding on 28 October (Figure 9.3l). Budwood was collected and stored during early winter from the same vineyard.

The trial was set up as a randomised split-plot design with the main plots consisting of four panels (12 vines) for each treatment, and sub-plots consisting of two panels (six vines) each for winter and spring cuts, with eight replicates, utilising 576 vines in four rows of the vineyard.

Vines were assessed for emergence of watershoots and graft success in spring (21 November; Figure 9.3m) and summer (16 February 2018).

In 2018, a further four adjacent rows (582 vines) in the same vineyard were cut just above the irrigation wire in spring (3 October), followed by painting all wounds with Greenseal. All stumps without watershoots were grafted on 23 November, using budwood collected from the vineyard in winter. On 8 February 2019, vines were assessed for emergence of watershoots and graft success.

In each year, 2019 and 2020, a further four adjacent rows were cut just above the irrigation wire in winter (3 and 9 July, respectively) and in spring (5 and 8 September, respectively), followed by painting all wounds with Greenseal. All stumps without watershoots were grafted on 9 and 29 October, respectively, using budwood collected from the vineyard in winter. Each trial was set up as a randomised block design with plots consisting of two panels (six vines) each for winter and spring cuts, and with 44 replicates, utilising 541 vines. On 21 January 2020 and 2 February 2021, vines were assessed for emergence of watershoots and graft success.

In 2021 a further four adjacent rows were cut just above the irrigation wire in winter (18 August) and in spring (26 October), followed by painting all wounds with Greenseal. Watershoots from 2020 were retained but no grafting treatments were undertaken. The trial was set up as a randomised block design with plots consisting of two panels (six vines) each for winter and spring cuts, and with 44 replicates, utilising 541 vines. On 19 January 2022, vines were assessed for presence of a watershoot retained from 2020, and total number of watershoots emerged.

The total number of trunks producing watershoots from each treatment were analysed using a binomial generalised linear model (GLM) with a logit link using Genstat® 19th Ed. A Wald test was used to determine significant differences between treatments, and the Bonferroni test was used to make multiple comparisons between treatments.



Figure 9.3. Remedial surgery vineyard trial in the Clare Valley, South Australia. Visual assessments of dieback (a) were conducted prior to remedial surgery on 21 June 2017. Watershoot induction treatments included hitting with a hammer (b), making an X-shaped cut with a tomahawk (c) and rubbing bark with a wire brush (d). Trunks were cut with a chainsaw below the cordon and above the irrigation wire (e-g) in both winter and in spring (27 September 2017), with cross-sectional staining revealed at the bottom (left) and top (right) of trunk (h-i). Wounds were painted with Greenseal™ (j) immediately following surgery and then Dormex® plant growth regulator was applied to designated trunks with a backpack sprayer (k). T-bud grafting was undertaken on 28 October (l). Shoots emerging from some stumps in spring 2017 on four rows of vines with trunks cut above the irrigation wire (m).

9.3 Results

9.3.1 Remedial surgery

Disease severity pre-remedial surgery

For the King Valley vineyard, the severity of dieback at the time of remedial surgery ranged from 13 to 16% (Figure 9.4a). Following remedial surgery, the severity of internal wedge-shaped staining in the cross-section of the trunks was 4, 7 and 10% for low, mid and high-cut vines, respectively. The severity of central staining in the cross-section of the trunks was 10, 22 and 22% for low, mid and high-cut vines, respectively. *Diplodia seriata* was the primary BD pathogen isolated from the representative symptomatic wood samples collected. No *Eutypa lata* was isolated from symptomatic samples while one sample tested positive to another ED pathogen, *Cryptovalsa ampelina*.

For the existing remedial surgery trial at Hilltops vineyard, the severity of dieback at the time of remedial surgery was higher than for the King Valley vineyard at 16 to 33% (Figure 9.4b). However, the severity of internal wedge-shaped and central staining in the cross-section of the trunks was lower at 0.6 to 2.8% and 2 to 6%, respectively. Botryosphaeriaceae were isolated from the representative symptomatic wood samples collected, while no *E. lata* was isolated from any of samples

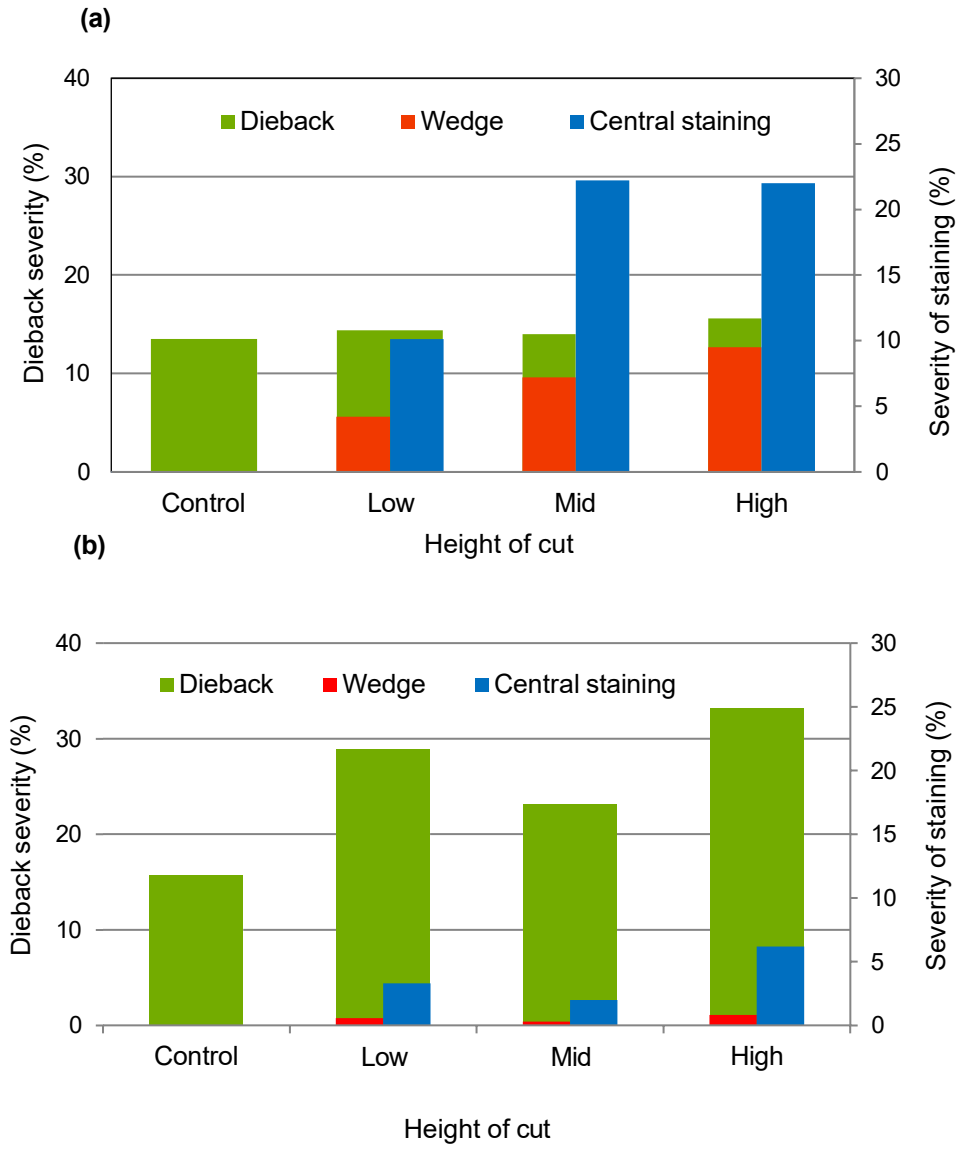


Figure 9.4. The severity of dieback assessed immediately prior to remedial surgery (green columns) and the severity of necrotic staining in the form of a wedge (red) or central (blue) staining around the pith assessed following remedial surgery for grafted vines in (a) King Valley, Vic and (b) Hilltops, NSW.

Shoot production

The first assessment for watershoot production following remedial surgery in the King Valley was in October 2018. Shoot production for low-cut vines was 57% in 2018 and decreased to 55% in 2021 (Figure 9.5a and Figure 9.6). Shoot production for mid-cut vines was 58% during the first season and increased to 63% in 2021. Shoot production for high-cut vines was 74% in 2018, reduced to 71% in 2019 and 2020 and increased to 76% 2021 (Figure 9.5a).

Shoot production for the existing remedial surgery trial at Hilltops using own-rooted vines, was significantly higher compared to the grafted vines at the King Valley with 97-100% shoot production in 2015 (Figure 9.5b). However, some of the reworked vines died with only 92-95% of the vines producing shoots in spring 2021.

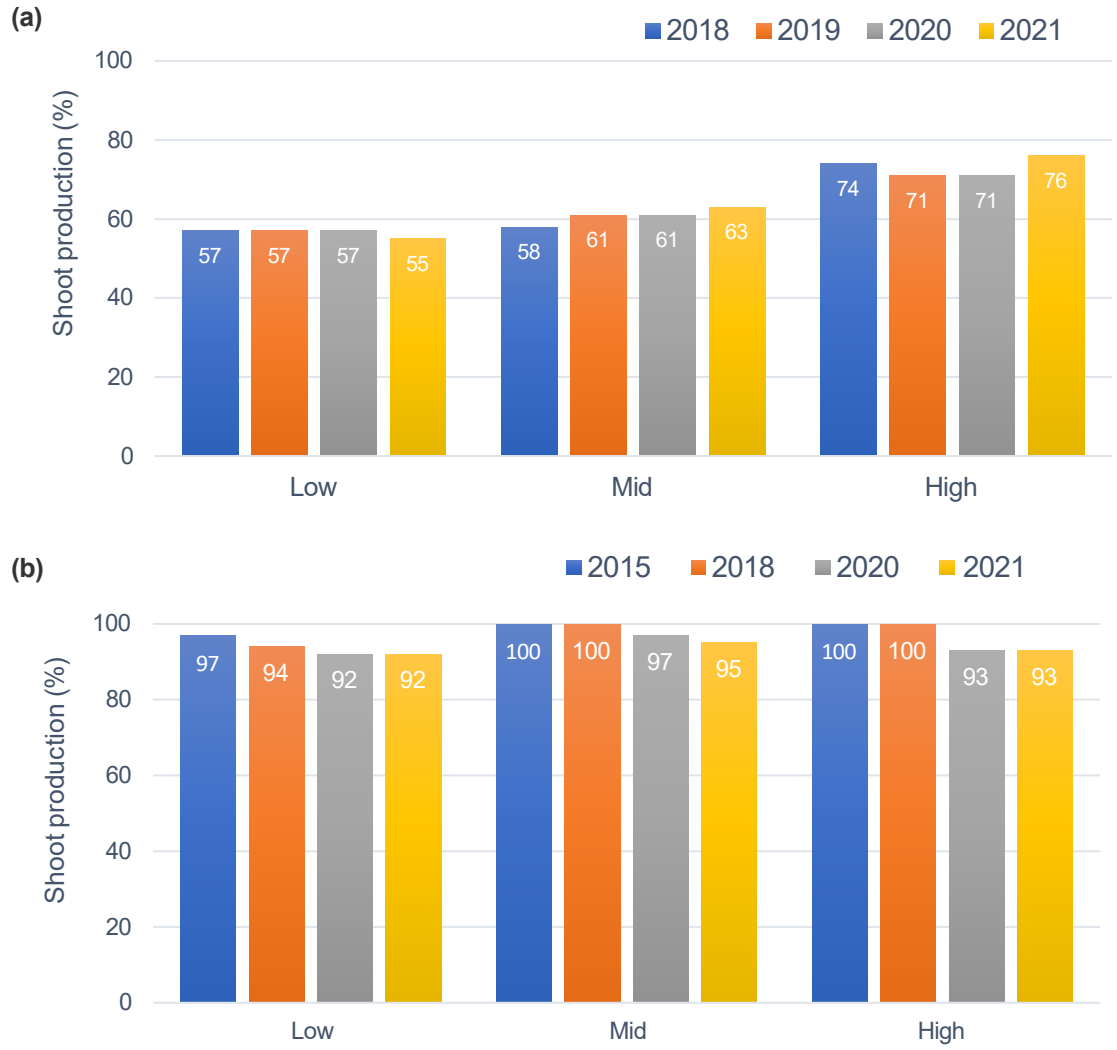


Figure 9.5. Production of water shoots in different growing seasons following remedial surgery for (a) King Valley, Vic and (b) Hilltops, NSW vineyards.

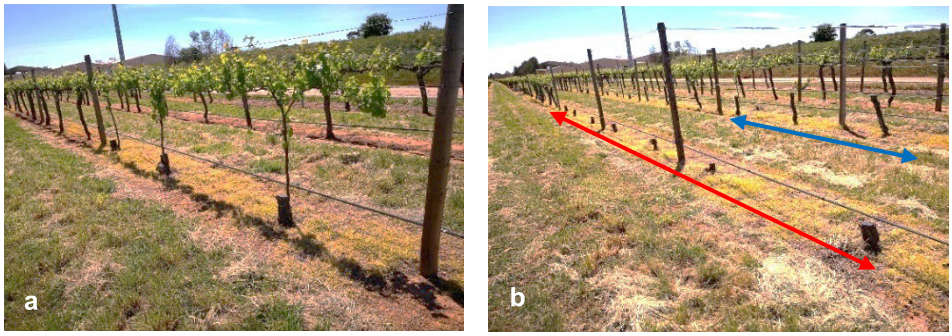


Figure 9.6. Watershoot production one year after remedial surgery in King Valley, Vic. a) Low-cut grafted vines with watershoots and retrained as new trunks and b) low (red arrow) and mid-cut (blue arrow) grafted vines without watershoots.

Disease severity post-remedial surgery

For the grafted vines in the King Valley, the initial severity of dieback in the uncut vines (non-treated controls) in 2017 was 14% and gradually increased to 27% by October 2021 (Figure 9.7). In 2021, dieback symptoms were first observed in 7%, 3% and 1% of the high, mid and low-cut vines, respectively, although the severity was relatively low ranging from 0.1 to 0.5%.

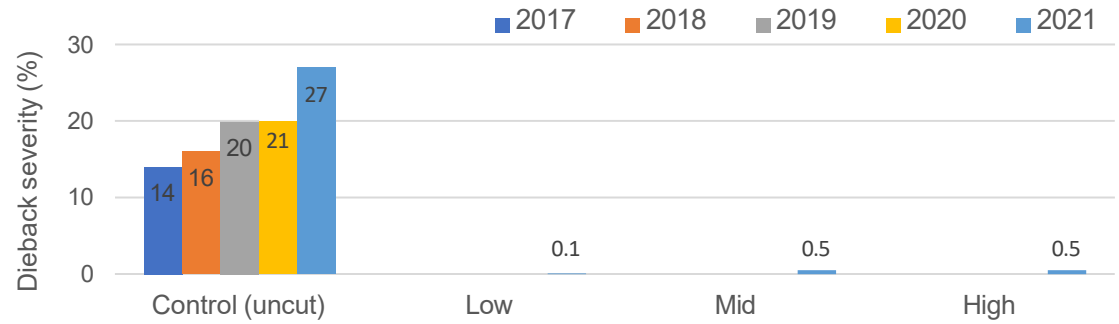


Figure 9.7. Assessment of dieback severity for non-treated control grafted vines (uncut) in King Valley, Vic from 2017 - 2021.

For the own-rooted vines in Hilltops, the initial severity of dieback in the uncut vines (non-treated control) was 19% in 2015, increased to 30% in 2018 and 2020, and 31% in 2021 (Figure 9.8). A greater severity of dieback was recorded in 2016, 2017 and 2019 (43-44%) which was most likely due to the assessments being done too early in the season, when shoots were still relatively small (data not shown).

In 2018, dieback symptoms were first observed in 40%, 15% and 5% of the high, mid and low-cut vines, respectively, although the severity was relatively low, ranging from 0.3 to 3% (Figure 9.8). In 2021, the incidence of dieback symptoms had increased to 53%, 25% and 33% for high, mid and low-cut vines, respectively, with severity ranging from 2-4%.

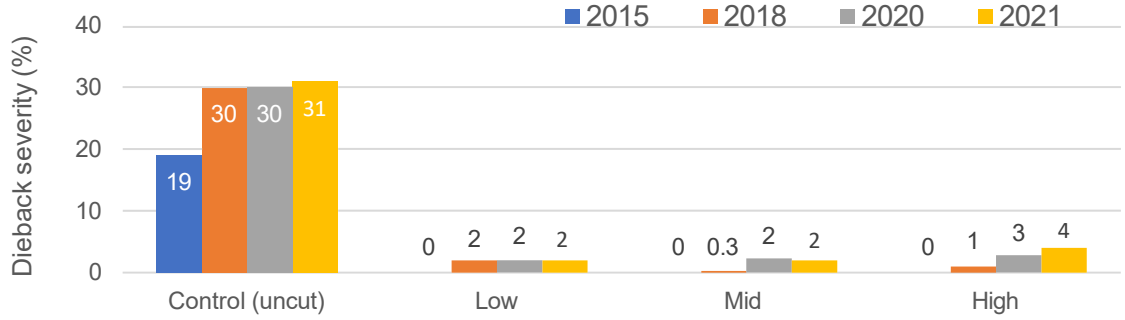


Figure 9.8. Assessment of dieback severity for own-rooted vines in Hilltops, NSW in 2015, 2018 and 2020 -

2021.

9.3.2 Watershoot induction

Prior to a mid-winter remedial treatment on 21 June 2017, vines were visually assessed with 100% incidence and 48% severity of GTD symptoms overall (Figure 9.3a). Disease severity was similar (*P*>0.05) in vine plots designated for cutting in winter (47%) and spring (48%), and between vine plots designated for each treatment (45-50%).

Cross-sectional cuts of trunks with a chainsaw revealed disease staining at the top of the excised trunk in 86% of vines and just above the irrigation wire in 27% of vines (Figure 9.3h-i). Overall, severity of staining was 20% and 4% at the top and bottom of the excised trunk, respectively. Severity of cross-sectional staining in the high cut was similar (*P*>0.05) in vine plots designated for cutting in winter (22%) and spring (18%), but differed (*P*<0.05) between vine plots designated for each treatment (14-27%). Severity of cross-sectional staining in the low cut was similar (*P*>0.05) in vine plots designated for cutting in winter (4.9%) and spring (3.6%), and between vine plots designated for each treatment (1.6- 5.5%).

Sections of trunk were sampled by drilling into the various types of staining, with small pieces of wood shavings placed onto PDA for incubation (Figure 9.9). Isolations confirmed the presence of *Eutypa lata*, *Phaemoniella chlamydospora* and a Botryosphaeriaceae sp. (Table 9.1). *E. lata* was most commonly isolated from dark, wedge shaped staining and *P. chlamydospora* was most commonly found in the small, round black spots.



Figure 9.9. Sections of trunk (1-5) collected from 22-year-old Cabernet Sauvignon vines in Clare Valley, South Australia, that were subjected to remedial surgery, showing drill-hole locations in cross-sectional staining, and small pieces of shavings yielding fungal cultures, following incubation on PDA (right).

Table 9.1. Pathogens isolated from trunk samples (Figure 9.9) collected from 22-year-old Cabernet Sauvignon vines in Clare Valley, South Australia, which were subjected to remedial surgery.

Section	Pathogen/s identified
1	B. *Phaeomoniella chlamydospora* C. *Eutypa lata*
2	A. *E. lata* and *P. chlamydospora*
3	*E. lata*
4	*E. lata*
5	B. *E. lata* C. Botryosphaeriaceae sp.

On 21 November 2017, watershoot production was assessed by scoring trunks for shoot growth from within the painted circle and outside the circle, and anywhere else on the trunk (Figure 9.10). Watershoots emerged within the circle from between 9 and 17% of trunks cut in winter and between 4 and 10% of trunks cut in spring, with no difference (*P*>0.05) between treatments. Outside of the

circles, watershoots emerged from between 74 and 85% of trunks cut in winter and between 55 and 79% of trunks cut in spring, with no difference between treatments. The majority of watershoots on trunks occurred outside the circle.

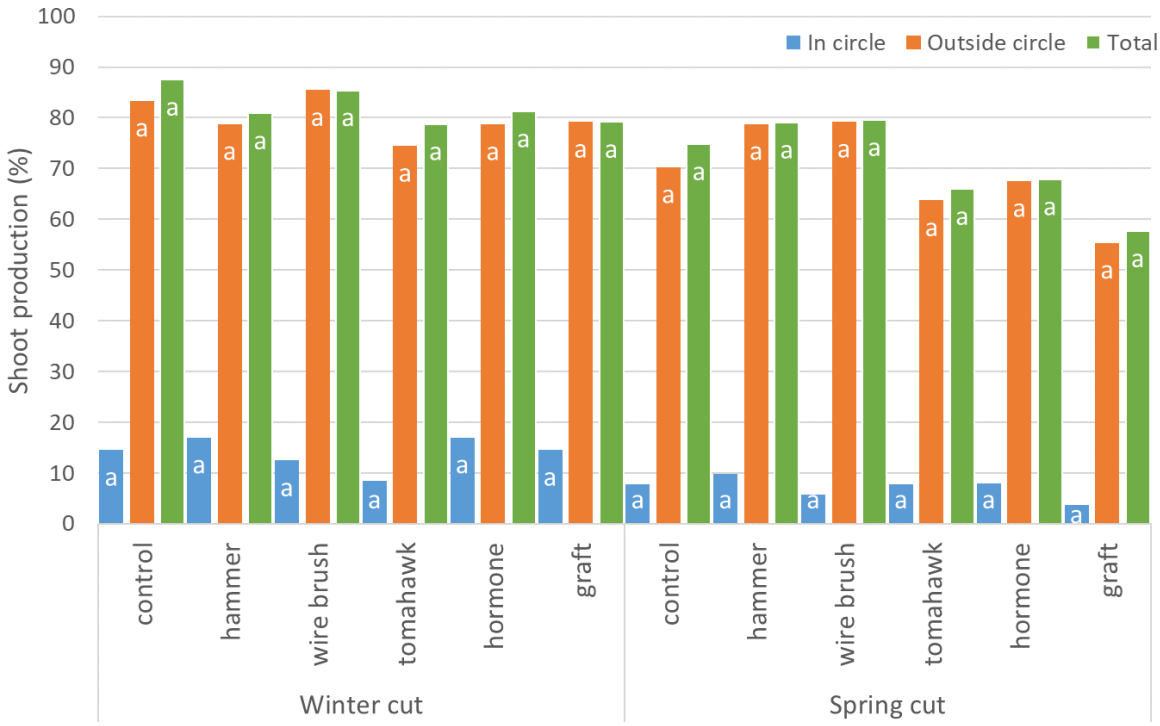


Figure 9.10. Watershoots emerging in spring (21 November 2017) from 22-year-old Cabernet Sauvignon trunks cut in winter and spring in a remedial surgery vineyard trial in Clare Valley, South Australia. Treatments included hitting with a hammer, making an X-shaped cut with a tomahawk, rubbing bark with a wire brush, a plant growth regulator treatment of Dormex and T-bud grafting. Shoots emerging from within the white circle where treatments were applied (blue columns) or from outside the circle elsewhere on the trunk (orange columns) and the combined total (green columns) are shown. Columns with letters in common are not significantly different from each other (*P*>0.05).

Watershoot and graft success was assessed on 16 February 2018. Watershoots emerged from 85 and 83% of untreated control trunks when cut in winter and spring, respectively, with no significant difference (*P*>0.05) between the times of remedial surgery (Figure 9.12). Grafted trunks were recorded with the highest percentage of success, 98 and 92% from winter and spring cuts, respectively, although this was not significantly greater (*P*>0.05) than untreated trunks due to large variation of data. The other four treatments ranged from 81-92%, with no significant difference (*P*>0.05) from untreated vines.

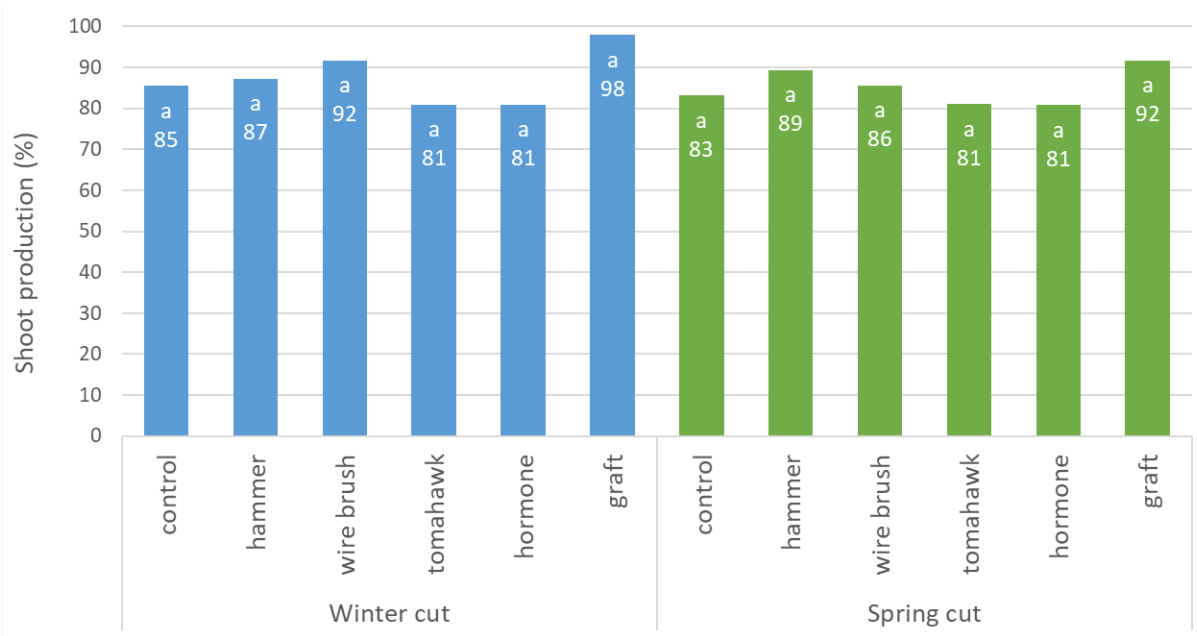


Figure 9.11. Shoot production from trunks in summer (16 February 2018) on 22-year-old Cabernet Sauvignon cut in winter (blue columns) and spring (green columns) in a remedial surgery vineyard trial in Clare Valley, South Australia. Treatments included hitting with a hammer, making an X-shaped cut with a tomahawk, rubbing bark with a wire brush, a plant growth regulator treatment of Dormex and T-bud grafting. Columns with letters in common are not significantly different from each other (*P*>0.05).

Figure 9.13 shows a summary of shoot production from vines cut in winter and spring during the years 2017 to 2021, comparing incidence of trunks with watershoots and the increased shoot production resulting from grafting. Watershoot production varied between years, but data could not be analysed due to differing experimental design between years. For winter cuts, watershoots emerged on 70-87% of trunks between 2017 and 2021. Grafting trunks without watershoots increased the overall shoot production by 11-13%, significantly (P<0.05) also in 2019 and 2020. For spring cuts, watershoots emerged on 71-87% of trunks between 2017 and 2021. Grafting trunks without watershoots increased shoot production by 6-19%, significantly (P<0.05) also in 2018, 2019 and 2020. Graft strike was relatively low in 2018 (31% in spring cuts) compared to 2019 (60% in winter cuts, 68% in spring cuts) and 2020 (78% in winter cuts, 77% in spring cuts). When comparing winter and spring cuts, there was slightly higher incidence of shoot production following winter cuts in 2017 and spring cuts in 2019, 2020 and 2021, although the differences were not significant (P>0.05), except following grafting in 2019. In 2021, with watershoots retained from the previous year and no grafting treatments, shoot production was recorded at 87 and 88% for winter and spring cuts, respectively.

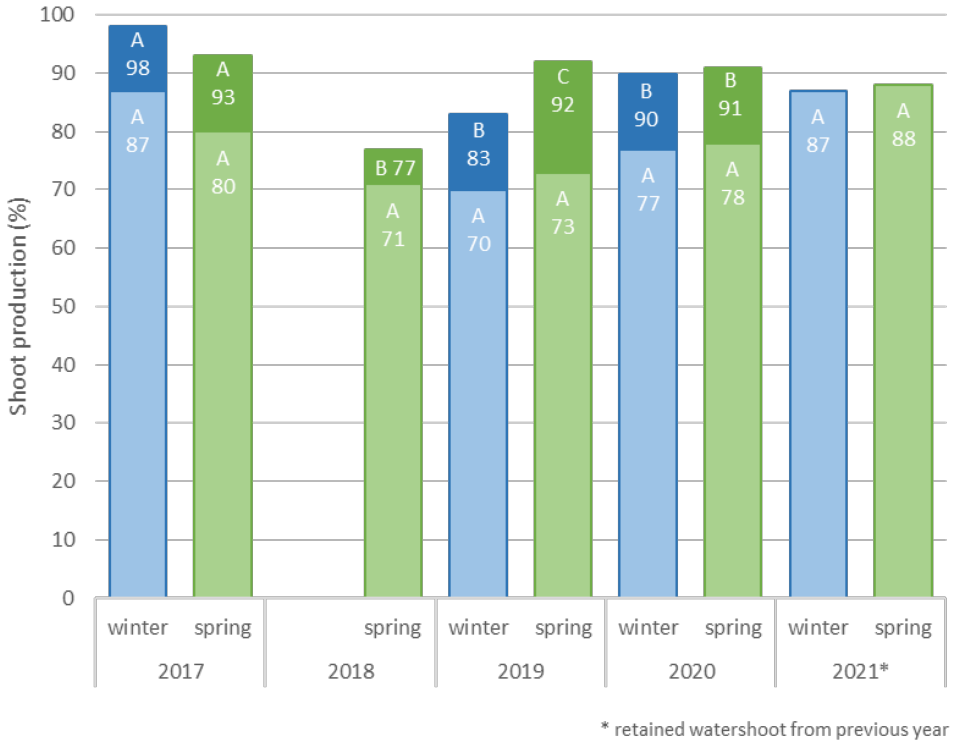


Figure 9.12. Summary of shoot production from trunks of Cabernet Sauvignon (planted 1995) when cut in winter (blue columns) and spring (green columns) over five years in a vineyard trial in the Clare Valley, South Australia. For each year (2017-2020), watershoot production from grafting (dark coloured columns) is shown. Within each year, columns with no letters in common are significantly different from each other (*P*<0.05).

9.4 Discussion

9.4.1 Remedial surgery

This study represents the first scientific evaluation of remedial surgery to control BD on both own-rooted and grafted vines. Remedial surgery or 'trunk renewal' has long been used in vineyards to control ED (Carter 1994; Creaser and Wicks, 2004) and BD (Leavitt 1990). More recently, it was shown to be successful in managing ED on own-rooted vines in South Australia (Sosnowski *et al*. 2011; SAR 06/01).

Similar to the results in SA, this study also showed that own-rooted vines can recover and produce new shoots after remedial surgery. However, grafted vines, particularly those that were cut near the graft union have lower shoot production compared to own-rooted vines. These results suggest that remedial surgery may be more successful in own-rooted vines than grafted vines.

In the existing remedial surgery trial at Hilltops, the severity of dieback in untreated vines increased by 12% after seven years of monitoring. Results from the King Valley vineyard also showed 13% increase in the severity of dieback after four years. This indicated that if infected vines were not reworked, the dieback severity would have increased by 2-3% each year. This increase in severity is relatively low compared to the remedial surgery trials conducted in two Hunter Valley vineyards (HV1 and HV2) where severity increases of 5-10% each year were reported, eventually leading to removal of the vineyards by the end of the project (SAR 1205; Sosnowski *et al*. 2017). The greater increase in severity for the Hunter Valley vineyards may be due to the higher disease severity at the time of surgery, which ranged from 74 to 77% for HV1 and 53 to 65% for HV2 vineyards, compared with only 15-30% disease severity at Hilltops. However, four years after remedial surgery, 42% of the reworked vines at Hilltops and 7% of the reworked vines in the King Valley started to exhibit dieback symptoms, although the severity was very low. These vines were not treated with annual pruning wound protection following remedial surgery, suggesting that new infections may have occurred. Thus, the application of pruning wound protectants during the pruning season is vital to ensure the long-term success of remedial surgery for the control of BD.

Remedial surgery has proven to be an effective method for managing ED and for increasing the longevity of vines. The preliminary data from these trials showed that this strategy may also be effective for managing BD. However, remedial surgery was found to be most successful in own-rooted vines. Thus, further work on grafted vines is necessary, and other options such as regrafting onto the scion (top-working) or directly onto the rootstock should also be considered. Development of other trunk disease management strategies for grafted vines is important, particularly for regions where phylloxera is considered a serious problem.

9.4.2 Watershoot induction

When vines were cut above the irrigation line over four years of this study, without intervention, watershoot growth from stumps ranged between 70 and 87%.

A watershoot induction trial was undertaken to evaluate five methods of improving watershoot growth following remedial surgery. This is the first known scientific evaluation of its kind. The low incidence of watershoots emerging from within the treatment circles, and the majority of watershoots emerging randomly from other parts of the trunks, indicates there was no influence of most treatments applied at the time of remedial surgery on the production of shoots. However, by summer, the grafting treatment had increased shoot production by 9-13%, the highest incidence of all treatments, albeit not statistically significant compared with the untreated controls.

To further evaluate the potential of grafting to increase regrowth following remedial surgery, four adjacent rows were subjected to remedial surgery in each of the following four years. In 2018-2020, grafting trunks that had not produced a watershoot significantly increased the incidence of trunks with shoot growth by 6 to 19%. Graft strike was relatively low (31-78%) over the four years of the trial compared to the industry standard of over 90% for top-working (T. Hoare pers. comm.). This is not unexpected, as trunks had not produced a watershoot naturally, and sap flow may have been compromised by trunk disease infection or other factors, preventing a successful graft union. Furthermore, in 2018 when grafting strike was only 31%, there were hot, dry conditions, which contributed to very poor graft success across the Clare Valley region in that spring period (T. Hoare pers. comm.). Nevertheless, the grafting treatment, which only needed to be applied to trunks without a watershoot, improved regrowth of the remediated block.

For the final year of the study in 2021, where available, watershoots were retained from 2020. Although no grafting was undertaken, shoot production was recorded at 87-88%, which was comparable to shoot production with grafting in previous years. This supports the strategy of retaining watershoots prior to conducting remedial surgery, but further evaluation of this technique in a randomised and replicated trial is necessary to confirm its success in improving shoot production.

9.5 References

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10 SUSCEPTIBILITY OF CLONES AND ROOTSTOCKS

10.1 Introduction

There is evidence of tolerance of some grapevine cultivars to trunk disease in Australia (SAR 1205; Sosnowski *et al.* 2016b, Sosnowski *et al.* 2017) and abroad (Gramaje *et al.* 2018). Visual assessment for symptoms of trunk disease in a mature germplasm cultivar collection revealed a marked range of symptom severity between cultivars. Further evaluation of disease progression, by inoculation of pruning wounds in the vineyard and on detached canes, confirmed that some cultivars appeared more tolerant to infection by ED and BD pathogens. There was some correlation between cane width, lignin content and colonisation, which was consistent with findings from previous research (Rolshausen *et al.* 2008, Pouzelet *et al.* 2014, Hamblin 2015).

A preliminary visual assessment of symptoms in a clonal germplasm collection at the SARDI Barossa Valley Research Centre, South Australia, indicated that the severity of trunk disease varied considerably amongst the clones of a number of cultivars (Sosnowski *et al.* 2017). In Spain, Berlanas *et al.* (2017) reported variation in susceptibility of 'Tempranillo' clones to the BD pathogen *Neofusicoccum parvum* in a detached cane assay.

Rootstock genotypes were screened for pathogen colonisation using detached canes, and varied in their susceptibility to colonisation by ED and BD pathogens (SAR 1205; Sosnowski *et al*. 2017). Several genotypes of *V. vinifera* (Riesling) x *V. cineria* were less susceptible than a Shiraz control. Billones-Baaijens *et al*. (2014) reported that Teleki 5C and SO4 rootstocks (*V. berlandieri* x *V. riparia*) were the most susceptible to BD pathogen infection among the six most common genotypes used in New Zealand.

The SARDI germplasm collection in the Barossa Valley consists of a broad range of clones of *V. vinifera* cultivars. This study utilised the collection and undertook visual assessment of symptoms of trunk disease severity in clones of the main cultivars grown in Australia. Promising clones were selected for further evaluation of pathogen colonisation in detached cane assays. Historical clone and rootstock trials planted by the Department of Agriculture, South Australia in association with growers and wine companies, provided an ideal opportunity for visual assessment of grapevine trunk disease in mature vines. Furthermore, the replicated trials have provided an opportunity to conduct a statistical evaluation of pathogen colonisation in scion material planted on a range of rootstocks.

This research aimed to identify resistant or tolerant clones and rootstocks, in order to provide recommendations for future plantings to reduce susceptibility to trunk diseases.

10.2 Methods

10.2.1 Field assessment

On 24 October 2017, vines in the SARDI clonal collection located at the Nuriootpa Research Centre, were visually assessed. Cultivars included Cabernet Sauvignon, Shiraz, Pinot Noir, Grenache, Chardonnay, Riesling, Sauvignon Blanc and Semillon. Vines were planted between 1969 and 1996, one panel of three vines per clone without any replication. Vines were cordon trained and spur pruned, with no specific strategies to control trunk diseases.

Clonal and rootstock trials were planted between 1982 and 1998 by the South Australian Department of Agriculture, in collaboration with grapegrowers and wine companies in South Australian regions. A stocktake identified 12 trials that remained in production (Figure 10.1). Each trial (other than CHPN2) was designed as a randomised block design with replication. Between 24 October and 24 November 2017, trials were visually assessed for trunk disease symptoms.

Table 10.1. List of historical clonal and rootstock trials established by the South Australian Department of Agriculture in commercial vineyards of various wine regions, which were still available for assessment in November 2017.

Clonal trials					
Code	**Location**	**Cultivar**	**Planted**	**Clones**	**Trial design**
CACH1	Summertown, Adelaide Hills	Chardonnay	1985	G9V7, F1V3 (OF), I10V1, I10V5, Mendoza	Single vine plots 20 replicates
CMCH1	Kuitpo, Adelaide Hills	Chardonnay	1989	I10V5, WA 6002, 1959/NX/EUROPE	Single vine plots 16 replicates
CMCS1	McLaren Vale	Cabernet Sauvignon	1982	R2V6 (Seven Hills Clare), R2V11 (Seven Hills Clare), 44 (ex Coonawarra Stage 1 1974), LC10 (ex Langhorne Creek Stage 1 1974), LC79 (1981 selections from Langhorne Creek), LC 81, LC82, LC83, LC84, 84 (46 – ex BVRC Variety Block), SA125 (AS 70.2351), G9V3 (IS 70.2217)	Single vine plots 12 replicates
CMME1	McLaren Vale	Merlot	1996	Q45-14, 6R, D3V14, 8R	Single vine plots 38 replicates
CHPN1/2	Charleston, Adelaide Hills	Pinot Noir	1998	114, 385, 521, 777, D5V12, G8V7, MV6	Blocks (40-50 vine rows)

Rootstock trials					
Code	**Location**	**Cultivar (clone)**	**Planted**	**Rootstocks**	**Trial design**
RBCH1	Barossa Valley	Chardonnay (IS-73-8127)	1980	1202, 1613, 1616, 101-14, 110 Richter, 420A, AxR, Harmony, K51-32, Kober 5BB, Salt Creek, Schwarzmann, SO4, St George, Teleki 5A, Own roots	Three vine plots Five replicates
RACH1	Flaxman Valley, Adelaide Hills	Chardonnay (n/a)	1993	Antav 13, Ruggeri 140, Schwarzmann, SO4, Teleki 5C	Three vine plots Eight replicates
RPSH1	Urrbrae	Shiraz (BVRC12)	1993	420A, K51-40, Ramsey, Ruggeri 140, Schwarzmann, SO4, Teleki 5C, Own roots	Four vine plots 11 replicates
RCSH1	Clare Valley	Shiraz (BVRC12)	1993	J17-48, J17-69, Paulsen 1103, Ramsey, Own roots	Three vine plots nine replicates
RSSH1	Coonawarra	Shiraz (BVRC12)	1986	1616, 101-14, 34EM, 420A, Teleki 5C 2136, Teleki 5C 8343, Teleki 5C 8344, SO4 8341, Petit Verdot, Own roots	Three vine plots 10 replicates
RSCS1	Coonawarra	Cabernet Sauvignon (LC10)	1988	1616, 101-14, 125AA, 34EM, 420A, 5C 8343, Riparia, SO4, Teleki 8B, Own roots	Three vine plots Eight replicates

Clone/rootstock trial						
Code	**Location**	**Cultivar**	**Planted**	**Clones**	**Rootstocks**	**Trial design**
RCCH1	Clare Valley	Chardonnay	1990	1959, G9V7, I10V1, I10V5	R140, R99, Ramsey, Own roots	Three vine plots 10 replicates

Vines were visually assessed for overall severity of trunk disease symptoms (ED and BD), which included the presence of at least two dead spurs, cordon dieback, trunk cankers and foliar symptoms of Eutypa dieback. Ratings were on a percentage scale (0% - non-symptomatic, 100% - dead; Figure 9.1). Data were subjected to analysis of variance and least significant difference (LSD) at the 5% level was used for all pairwise comparisons, with standard error of the means calculated. Incidence of ED foliar symptoms was also recorded for the historical vineyard clone and rootstock trials by counting the number of vines with symptoms. All data were plotted as bar graphs.

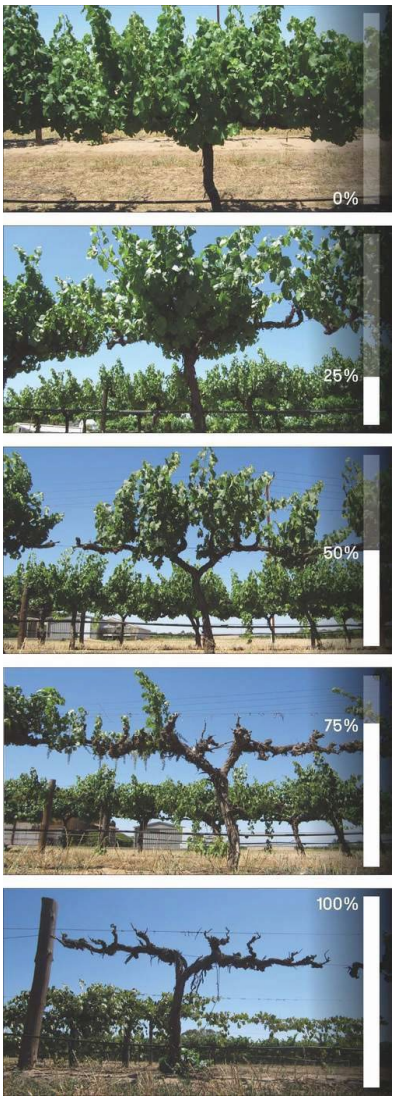


Figure 10.1. Rating scale for grapevine trunk disease dieback severity.

10.2.2 Greenhouse clone evaluation

Fifty clones of seven cultivars were selected for further evaluation from the SARDI clonal collection located at the Nuriootpa Research Centre (Table 10.2). For the more susceptible cultivars: Cabernet Sauvignon, Shiraz, Sauvignon Blanc and Sémillon, the two most symptomatic clones based on visual assessment were compared with two or three clones having the least symptoms. For the less susceptible cultivars: Chardonnay, Pinot Noir and Riesling, the three most symptomatic clones were compared with seven clones having least symptoms. In winter 2020 and 2021, canes were collected and stored in a cool room until commencement of evaluation experiments.

On 4 November 2020, a series of detached cane assay (DCA) experiments were established using methods described in Ayres *et al.* (2011). Canes of each clone were cut into 30 cm sections with the top surface 1 cm above the node and placed into holes made in 20 mm thick polystyrene boards (500 x 350 mm), ensuring that the bottom of the canes extended at least 1 cm below the boards (Figure 10.2a-b). Boards were set up to hold 5 x 8 canes placed in rows across the boards, with two boards per cultivar, or pair of cultivars for Cabernet Sauvignon/Shiraz and Sauvignon Blanc/Sémillon, placed adjacent to each other (Figure 10.2c). Each row represented a replication and columns contained each of the 10 randomly placed clones in a randomised block design with eight replications. Two extra boards were set up with two canes of each clone as controls. The boards with canes were floated on tap water in plastic tubs in a greenhouse maintained at approximately 25°C.

On the same day, wounds were inoculated with 200 spores of *E. lata* suspended in 20 µL SDW and applied using a pipette. Controls were not inoculated (NIC). *E. lata* spores were obtained using the methods described in Sosnowski *et al*. (2017; SAR1205). Water was changed in the plastic tubs monthly for the duration of the experiment.

Six months after inoculation on 1 April 2021, canes were removed from boards and processed as described in Sosnowski *et al*. (2017; SAR 1205). Cane diameter was measured with Vernier calipers midway between two nodes and recorded. Bark was removed from each cane using a sharp knife. The distance of discoloured lesions from the wound inoculation site was measured and recorded. The exposed wood was surface sterilised in 2.5% sodium hypochlorite (NaOCl) for 10 minutes and then washed twice in SDW. Using flame-sterilised secateurs, wood pieces (~2 mm discs) were taken at 5 mm intervals up to 100 mm below the inoculated wound and transferred onto PDA amended with antibiotic (streptomycin sulfate 100 mg/L). Samples were incubated at 25°C under fluorescent light for 12 h each day for seven days and the distance at which *E. lata* was reisolated below the point of inoculation recorded.

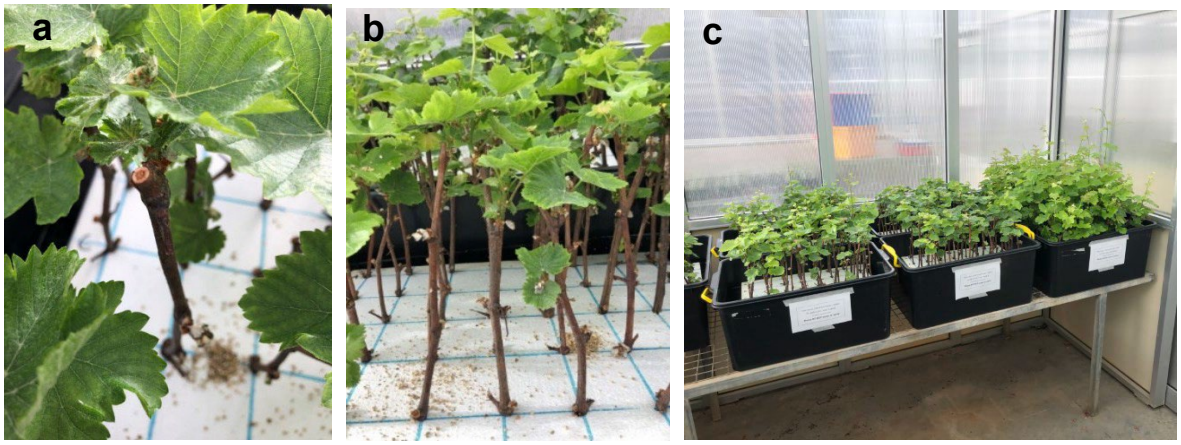


Figure 10.2. Detached cane assay in the greenhouse used to evaluate grapevine clones for susceptibility to eutypa and Botryosphaeria dieback pathogens. (a) wounds were made 1 cm above nodes, (b) canes were placed into holes in polystyrene sheets and (c) clones of each cultivar were randomly placed in two plastic tubs.

On 5 August 2021, the experiment was repeated, but wounds were inoculated with 200 spores of *N. luteum*. Six months after inoculation on 3 February, canes were processed as for the *E. lata* experiment, with colonisation distance of *D. seriata* assessed on PDA plate isolations.

A Shapiro-Wilk test was used to confirm if data were normally distributed, and then data were subjected to analysis of variance using R Studio (R Core Team 2021, Nielsen *et al*. 2021). Tukey's multiple comparison test was used to determine whether pathogen recovery distance and lesion lengths were significantly different from one another.

Correlation between pathogen recovery distance, lesion length and cane diameter were determined using regression analysis (Microsoft Excel).

10.2.3 Field rootstock evaluation

The rootstock trial RPSH1 (Table 10.1) located in Urrbrae, was used to further evaluate the susceptibility of scion (Shiraz clone BVRC12) on a range of rootstocks 420A, K51-40, Ramsey, Ruggeri 140, Schwarzmann, SO4, Teleki 5C, and on own roots. Using methods similar to those described in Sosnowski *et al*. (2017; SAR 1205) for cultivar susceptibility evaluations, one-year-old canes were pruned, using secateurs, to at least 30 cm length on 9 July 2020. Wounds were moistened by spraying with SDW immediately prior to inoculation with approximately 200 spores of either *E. lata* or *D. seriata* suspended in 20 µL SDW and applied using a pipette. Ten canes per vine were inoculated with each pathogen. The trial was a randomised block design, with two vine plots (one per pathogen) and eight treatments (rootstocks and own roots) per row, spread across five rows (replications).

Canes were removed from vines on 10 June 2021 and placed in cool storage. To determine the extent to which *E. lata* and *D. seriata* colonised the stems, bark was removed from canes using a sharp knife, and the extent of discoloured lesion measured and recorded, before canes were surface sterilised in 2.5% sodium hypochlorite for 10 minutes followed by rinsing in sterile distilled water. Wood pieces (~2 mm discs) were taken at 10 mm intervals up to 200 mm below the inoculated wound and transferred to PDA. Following 7-14 days of incubation at 23ºC under fluorescent light, cultures were identified using colony morphology, and the distances at which fungi were reisolated below the point of inoculation recorded.

A Shapiro-Wilk test was used to confirm if data were normally distributed, and then data were subjected to analysis of variance using R Studio (R Core Team 2021, Nielsen *et al*. 2021). Tukey's multiple comparison test was used to determine whether pathogen recovery distance and lesion lengths were significantly different from one another.

10.3 Results

10.3.1 Field assessment

10.3.1.1 Clones

Germplasm collection

The severity of dieback symptoms varied substantially for clones of each cultivar assessed. For the more susceptible cultivars (Cabernet Sauvignon, Shiraz, Grenache and Sauvignon Blanc) most clones had greater than 50% mean dieback severity (Figure 10.3 and Figure 10.4). For the less susceptible cultivars (Pinot Noir, Chardonnay, Riesling and Semillon) most clones had less than 50% mean dieback severity.

Cabernet Sauvignon: Clone 12R had fewer dieback symptoms (35%) than all other clones in the collection (60-100%), although clone 12R vines were only 23 years of age with most other clones being older (Figure 10.3). Clone 10R, also 23 years old, was recorded with 60% dieback severity.

Shiraz: Clone SA 41 had fewer dieback symptoms (45%) than most other clones (56-95%; Figure 10.3). The age of clone SA 41 was unknown.

Pinot Noir: Clones ranged from 9-52% dieback severity (Figure 10.3). When comparing clones of known age (31 years), Bourgogne H80A and H140A had 10% dieback severity compared with 40-50% severity in clones Cortaillod H120B, MV 6 H170A and Oberlin H120A.

Grenache: All clones ranged from 67 to 96% dieback severity (Figure 10.3).

Chardonnay: Clones IC.85.8498, G9V5, Q949-08 (Cl 277) and Q949-03 (Cl 76) were recorded with <6% dieback severity, with vine age ranging from 21-26 years (Figure 10.4). Clone 6002 ex WA (33 years old) had less dieback (14%) than clones Penfolds 1959 (36%) and C (white) (39%), which were both 29 years of age.

Riesling: Clones GM 110, 812 H120B and 356 Trier, aged 25-27 years, had no dieback symptoms (Figure 10.4). All other clones ranged from 1-50% dieback severity.

Sauvignon Blanc: Clones H5V11 and F7V7 had fewer dieback symptoms (60%) than all other clones (73-100%), and in particular H5V10 and H5V12, which were the same age (27 years) but were dead (Figure 10.4).

Semillon: Clones LRC 147 and SA 147 had <10% dieback severity, compared with most other clones ranging from 12-62% (Figure 10.4). The age of many clones was unknown.

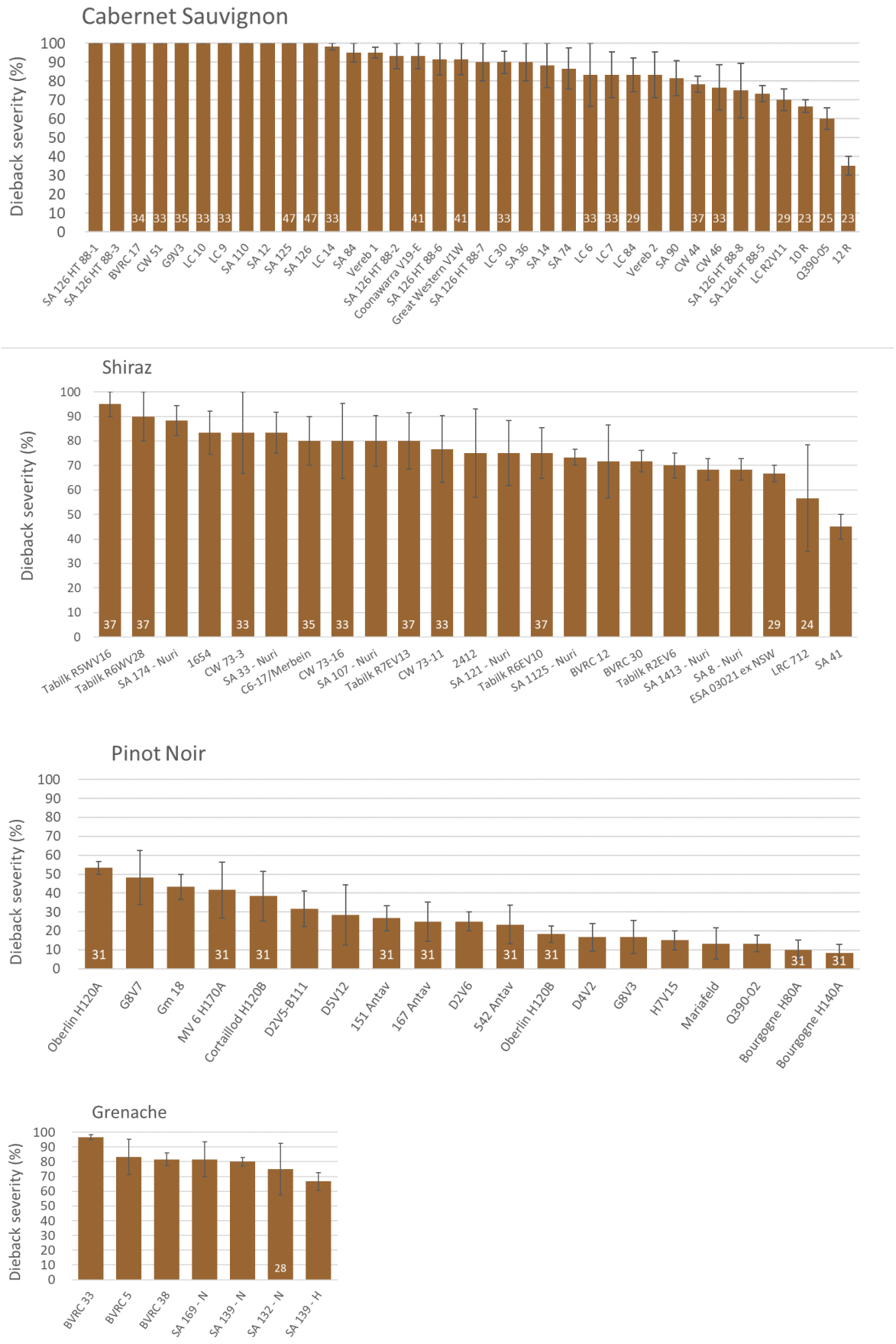


Figure 10.3. Mean dieback severity in clones of various red wine cultivars planted at the Nuriootpa Research Centre germplasm collection Barossa Valley, South Australia and assessed in October 2017. Age of vines, where known, is indicated at the base of columns for each clone. Figures are based on three vine plots that are not replicated. Bars represent standard error of the mean.

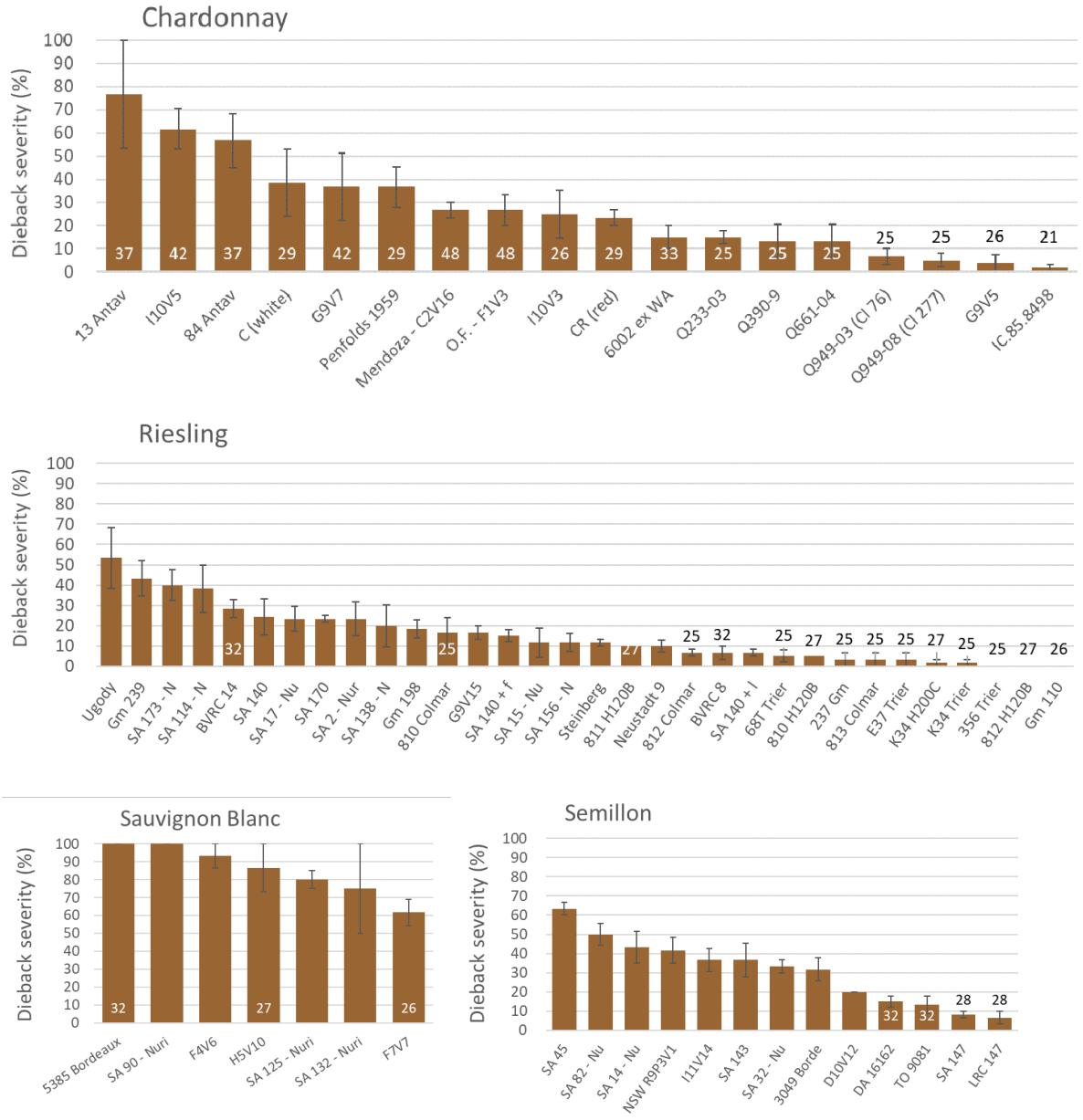


Figure 10.4. Mean dieback severity in clones of various white wine cultivars planted at the Nuriootpa Research Centre germplasm collection Barossa Valley, South Australia and assessed in October 2017. Age of vines, where known, is indicated at the base of columns for each clone. Figures are based on three vine plots that are not replicated. Bars represent standard error of the mean.

Clonal trials

Chardonnay: In the two trials assessed, dieback severity ranged from 30-44% (Summertown) and 13-18% (Kuitpo), with no significant differences (P>0.05) between clones within each trial (Figure 10.5). Incidence of ED foliar symptoms were observed to be greater for clone F1V3 and I10V5 than other clones in Summertown and Kuitpo, respectively.

Cabernet Sauvignon: Clones LC82, LC79, LC83 had 46-50% dieback severity, significantly lower (P<0.05) than that of clones 84 and R2V6 with 72-75% dieback severity (Figure 10.6. Conversely, it was observed that 82% of clone LC82 vines had eutypa dieback foliar symptoms and clone 84 had only 25%.

Merlot: The four clones ranged from 30-37% dieback severity, with 8R recorded with significantly less (P<0.05) dieback than D3V14 (Figure 10.6). Foliar symptoms were low (up to 10%) for all four clones.

Pinot Noir: Dieback severity and foliar symptom incidence was low for all clones in the trial (Figure 10.7). Incidence of vines with dieback ranged from 15-44%. Clone D5V12 was observed with the most symptoms and clone 385 the least, but data could not be statistically analysed.

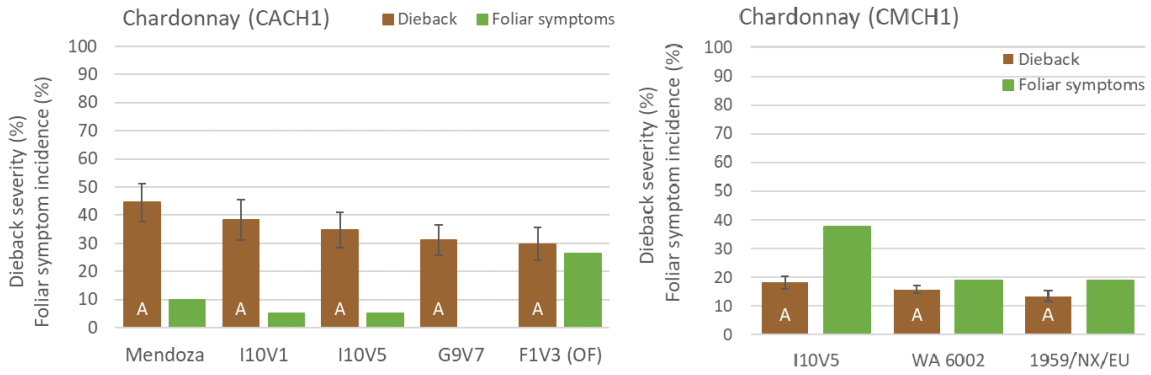


Figure 10.5. Mean dieback severity (brown columns) and incidence of vines with Eutypa dieback foliar symptoms (green columns) on Chardonnay clones in trials within commercial vineyards in Summertown, (CACH1, planted 1985) and Kuitpo (CMCH1, planted 1989) in Adelaide Hills, South Australia. Trials were arranged as randomised block designs, with 20 and 16 replicates, respectively, and clones planted as single vine plots. Assessments were conducted in November 2017. For dieback severity, columns with no letters in common are significantly different from each other (P<0.05) and error bars represent standard errors of the means.

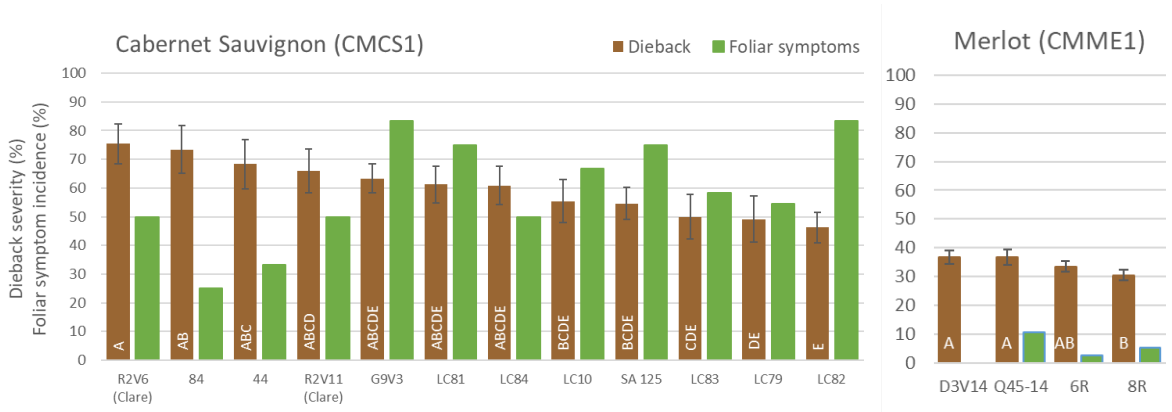


Figure 10.6. Mean dieback severity (brown columns) and incidence of vines with eutypa dieback foliar symptoms (green columns) on Cabernet Sauvignon and Merlot clones in trials within commercial vineyards in McLaren Vale, South Australia (CMCS1, planted 1982, CMME1, planted 1996). Trials were arranged as randomised block designs, with 12 and 38 replicates, respectively, and clones planted as single vine plots. Assessments were conducted in November 2017. For dieback severity, columns with no letters in common are significantly different from each other (P<0.05) and error bars represent standard errors of the means.

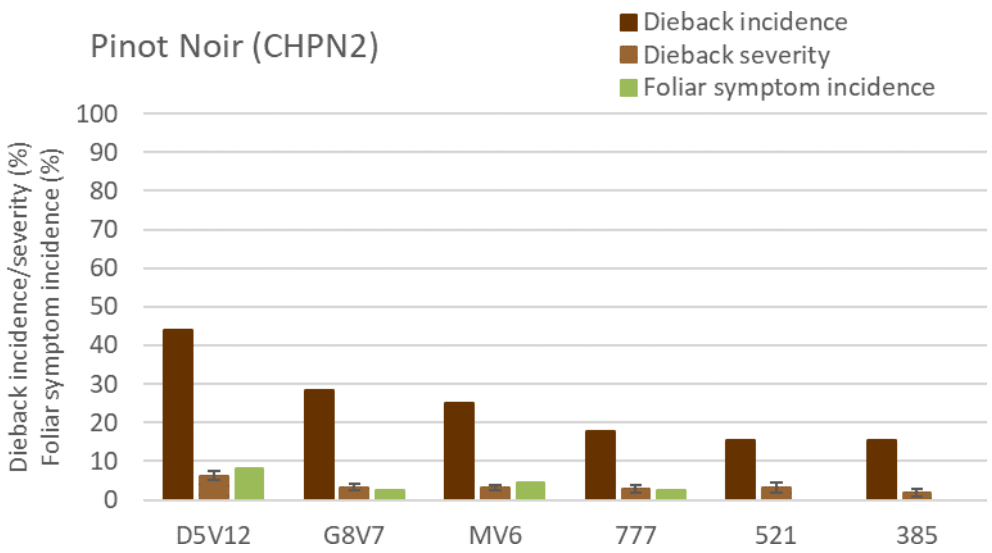


Figure 10.7. Mean dieback incidence (dark brown columns), severity (light brown columns) and incidence of vines with Eutypa dieback foliar symptoms (green columns) on Pinot Noir clones planted in multi-row blocks without randomisation or replication within a commercial vineyard in Charleston, Adelaide Hills, South Australia (CHPN2, planted 1998). Assessments were conducted on a single row (40-50 vines) within each clone block in November 2017. Bars represent standard error of the mean for dieback severity.

10.3.1.2 Rootstocks

Rootstock trials

Chardonnay: Dieback severity ranged from 1-30% for vines on all rootstocks in the Barossa Valley trial, with rootstock 1616 and Richter 110, having significantly less (P<0.05) symptoms than vines on Harmony, A x R, 1202 and 101-14 (Figure 10.8). Incidence of foliar symptoms varied from 0-38%, but was not always consistent with dieback severity. Vines on Harmony were observed with the most foliar symptoms and no symptoms were observed in any vines on 1616 or Kober 5BB.

In the Flaxman Valley trial, dieback severity ranged from 20-44%, with vines (clone Antav 13) on own roots having significantly lower (P<0.05) dieback severity than vines on the three rootstocks, and Richter 99 and 110 with the most dieback (Figure 10.8). Incidence of foliar symptoms was low for vines on all three rootstocks and own rooted vines.

Shiraz: Dieback severity varied between vines on rootstocks for the three trials assessed (Figure 10.9). In two trials (Urrbrae and Clare Valley), vines (clone BVRC 12) on own roots had a higher dieback incidence than most vines on rootstocks. Dieback severity ranged from 10-28% in Urrbrae and 53-70% in Clare Valley, with vines in both locations planted in 1993. Incidence of ED foliar symptoms ranged from 10-52% in Urrbrae, with most symptoms observed in vines on Ruggeri 140, Ramsey and on own roots. In Clare Valley, incidence of foliar symptoms ranged from 48-67%, with most symptoms observed in vines planted on own roots.

In the Coonawarra trial, dieback severity was similar across vines on most rootstocks and own roots (30-37%), although significantly more (P<0.05) dieback was recorded in vines on Teleki 5C 8343 (41%) and on Petit Verdot (81%). Incidence of foliar symptoms ranged from 17-38%.

Cabernet Sauvignon: Dieback severity ranged from 36-60% for vines on all rootstocks and own roots (Figure 10.10). Vines planted on Riparia rootstock had significantly greater (P<0.05) dieback severity than vines on all other rootstocks and own roots. Incidence of foliar symptoms ranged from 4-27%, with highest incidence observed in vines on 420A and own roots.

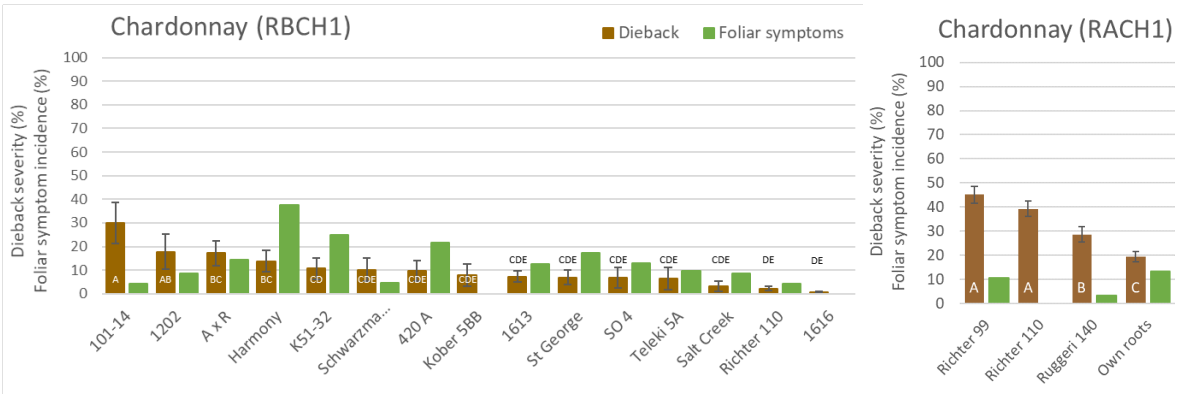


Figure 10.8. Mean dieback severity (brown columns) and incidence of vines with Eutypa dieback foliar symptoms (green columns) on Chardonnay planted on various rootstocks in trials within commercial vineyards in Barossa Valley (RBCH1, clone IS-73-8127 planted 1980) and Flaxman Valley, Adelaide Hills, South Australia (RACH1, clone Antav 13 planted 1993). Trials were arranged as randomised block designs, with 5 and 8 replicates, respectively, and rootstocks planted as three vine plots. Assessments were conducted in November 2017. For dieback severity, columns with no letters in common are significantly different from each other (*P*<0.05) and error bars represent standard errors of the means.



Figure 10.9. Mean dieback severity (brown columns) and incidence of vines with Eutypa dieback foliar symptoms (green columns) on Shiraz (clone BVRC 12) planted on various rootstocks in trials within commercial vineyards in Urrbrae (RPSH1, planted in 1993), Clare Valley (RCSH1, planted in 1993) and Coonawarra (RSSH1, planted in 1986), South Australia. The trials were arranged as randomised block designs, with 11, 9 and 10 replicates, respectively and rootstocks planted as four, three and three vine plots, respectively. Assessments were conducted in November 2017. For dieback severity, columns with no letters in common are significantly different from each other (*P*<0.05) and error bars represent standard errors of the means.

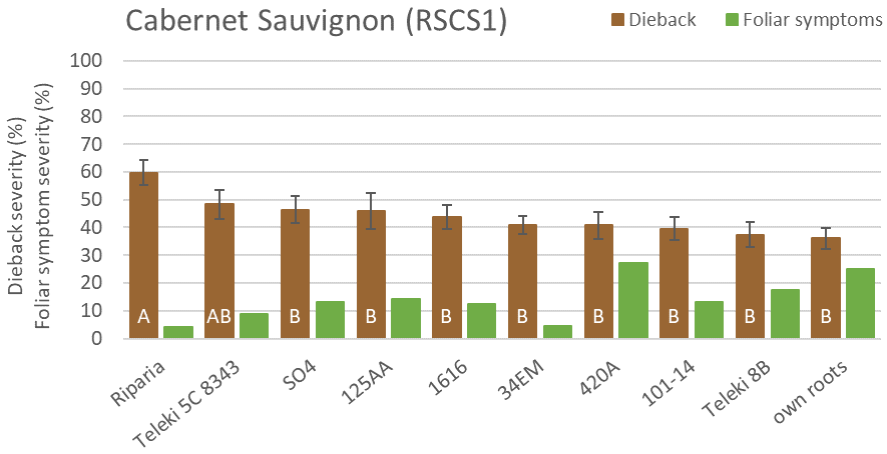


Figure 10.10. Mean dieback severity (brown columns) and incidence of vines with Eutypa dieback foliar symptoms (green columns) on Cabernet Sauvignon (clone LC 10) planted on various rootstocks in a trial within a commercial vineyard in Coonawarra, South Australia (RSCS1, planted in 1988). The trial was arranged as a randomised block design, with eight replicates, and rootstocks planted as three vine plots. Assessments were conducted in November 2017. For dieback severity, columns with no letters in common are significantly different from each other (*P*<0.05) and error bars represent standard errors of the means.

10.3.1.3 Clones and rootstocks

Dieback severity varied between combinations of Chardonnay clones on various rootstocks in a trial planted in the Clare Valley (Figure 10.11). The greatest severity of dieback was recorded on clone G9V7 planted on rootstock Richter 99 (62%), and the least on clone I10V5 planted on own roots (33%).

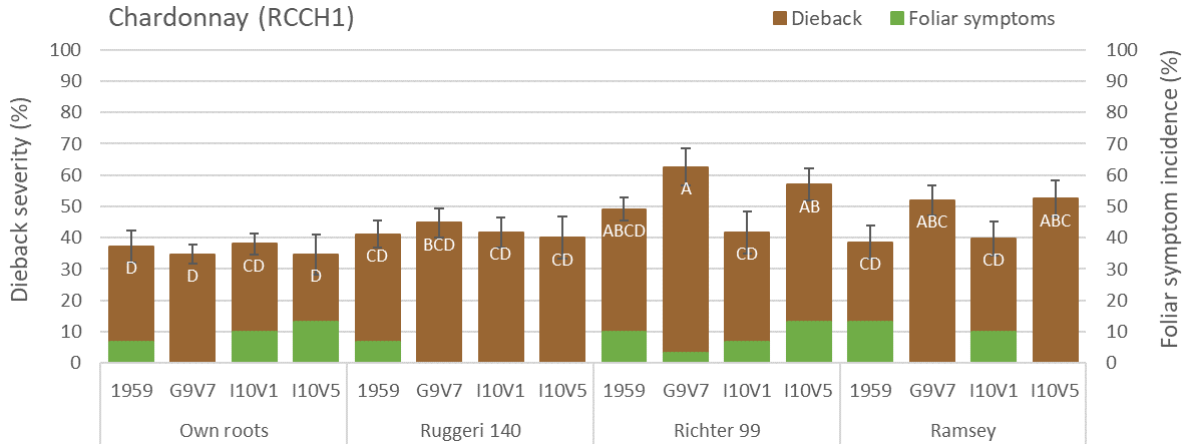


Figure 10.11. Mean dieback severity (brown columns) and incidence of vines with Eutypa dieback foliar symptoms (green columns) on various Chardonnay clones planted on various rootstocks in a trial within a commercial vineyard in Clare Valley, South Australia (RCCH1, planted in 1990), The trial was arranged as a randomised block design, with 10 replicates, and scion/rootstock combinations planted as three vine plots. Assessments were conducted in November 2017. For dieback severity, columns with no letters in common are significantly different from each other (*P*<0.05) and error bars represent standard errors of the means.

Statistical analysis revealed there was no interaction (*P*>0.05) between clones and rootstocks, so clones and rootstocks were analysed separately for individual effects (Figure 10.12). When comparing clones, dieback severity ranged from 40-49%, with clone G9V7 having significantly more (*P*<0.05) dieback than clone I10V1. Conversely, G9V7 was observed with lower incidence of eutypa dieback foliar symptoms (2%) compared with the three other clones (27-37%). Vines on own roots had significantly less (*P*<0.05) dieback (35%) than rootstocks Ramsey and Richter 99 (45-52%). Foliar symptom incidence was observed to be similar in own rooted vines and those on Richter 99 and Ramsey (22-32%), with Ruggeri 140 observed with only 7%.

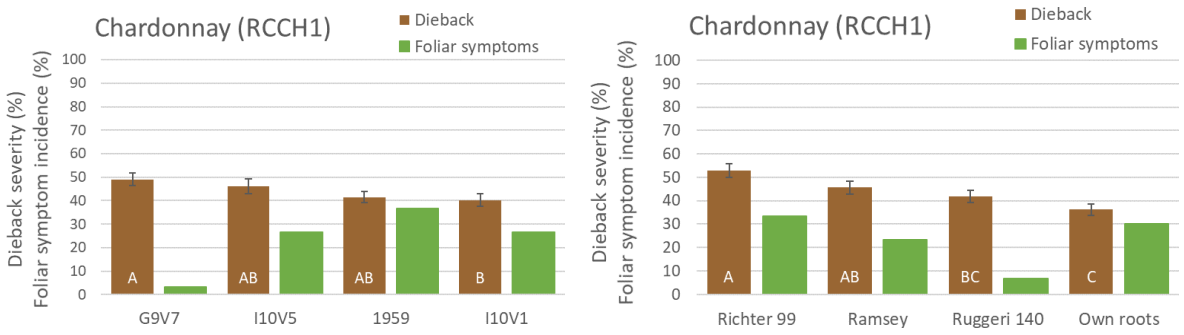


Figure 10.12. Mean dieback severity (brown columns) and incidence of vines with Eutypa dieback foliar symptoms (green columns) on various Chardonnay clones (left) and various rootstocks (right) in a trial within a commercial vineyard in Clare Valley, South Australia (RCCH1, planted in 1990), The trial was arranged as a randomised block design, with 10 replicates, and scion/rootstock combinations planted as three vine plots. Assessments were conducted in November 2017. For dieback severity, columns with no letters in common are significantly different from each other (*P*<0.05) and error bars represent standard errors of the means.

10.3.2 Greenhouse clone evaluation

Eutypa dieback

In the ED DCA, *E. lata* was recovered from canes of all cultivars, mean distances of which ranged between 46 and 64 mm (Cabernet Savignon), 26 and 46 mm (Shiraz), 53 and 71 mm (Sauvignon Blanc), 24 and 63 mm (Sémillon), 43 and 84 mm (Chardonnay), 2 and 46 mm (Pinot Noir) and 49 and 78 mm (Riesling) from the wound site over the six months incubation period (Figure 10.13). *E. lata* was not recovered from non-inoculated canes (data not shown). There was large variation in the data, evidenced by the standard error bars, and no significant differences (*P*>0.05) were found between clones within any cultivars.

The range of mean lesion lengths of lesions in canes inoculated with *E. lata* for each cultivar crosses over with the range for NICs (Table 10.2, Figures 10.13 and 10.14). The correlation between lesion lengths for canes inoculated with *E. lata* and NICs was R^2=0.4 (data not shown). There was no significant difference (*P*>0.05) between clones of Cabernet Sauvignon, Shiraz, Sauvignon Blanc, Sémillon and Chardonnay for length of lesions when inoculated with *E. lata* (Figure 10.13). For Pinot Noir clone 'D4V2', lesion length was greater (*P*<0.05) than that in some other clones. For Riesling clone 'Ugody', lesion length was greater (*P*<0.05) than for all other clones.

Table 10.2. The range of mean lesion lengths for clones of each cultivar when inoculated with *Eutypa lata, Diplodia seriata* and for non-inoculated controls (NIC) taken from Figures 10.13, 10.14 and 10.15.

Cultivar	Lesion length (mm)		
	E. lata	*D. seriata*	NIC
Cabernet Sauvignon	15-17	13-21	15-37
Shiraz	16-20	17-19	13-21
Sauvignon Blanc	15-36	14-26	20-38
Sémillon	12-16	12-20	15-20
Chardonnay	18-30	14-39	16-46
Pinot Noir	16-64	23-61	16-55
Riesling	17-37	17-33	19-66

For all cultivars apart from four clones of Pinot Noir, pathogen recovery exceeded the lesion length by between 29 and 49 mm (Cabernet Sauvignon), 6 and 27 mm (Shiraz), 22 and 55 mm (Sauvignon Blanc), 12 and 48 mm (Sémillon), 25 and 65 mm (Chardonnay), 1 and 19 mm (Pinot Noir) and 25 and 62 mm (Riesling; Table 10.3). There was no correlation between pathogen recovery distance and lesion length for any cultivars (R^2 < 0.06; data not shown).

The mean cane diameter ranged for clones of Cabernet Sauvignon (6.8 to 7.3 mm), Shiraz (6.0 to 7.8 mm), Sauvignon Blanc (5.0 to 7.1 mm), Sémillon (5.1 to 7.0 mm), Chardonnay (3.5 to 5.8 mm), Pinot Noir (3.4 to 6.3 mm) and Riesling (5.1 to 6.3 mm; Table 10.2). In general, mean cane diameter was

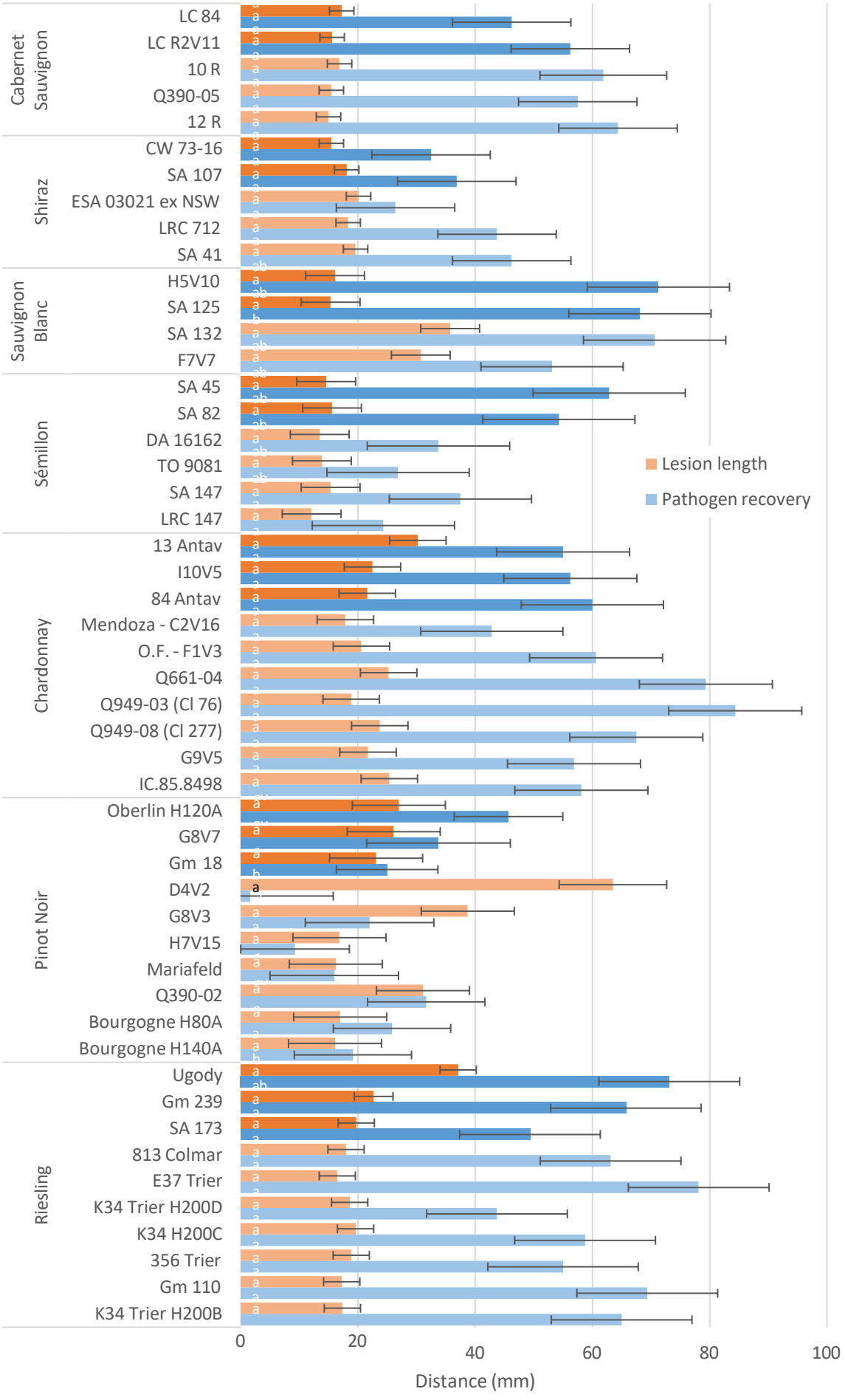


Figure 10.13. Recovery distance of *Eutypa lata* (blue columns), lesion length (orange columns) six months after inoculation of pruning wounds on detached canes of selected clones and cultivars from the clone collection at Nuriootpa Research Centre. Dark coloured columns represent clones with greatest dieback severity from visual assessments, and light columns the least. Columns with no letters in common are

significantly different from each other (P<0.05) and bars represent standard errors of the means.

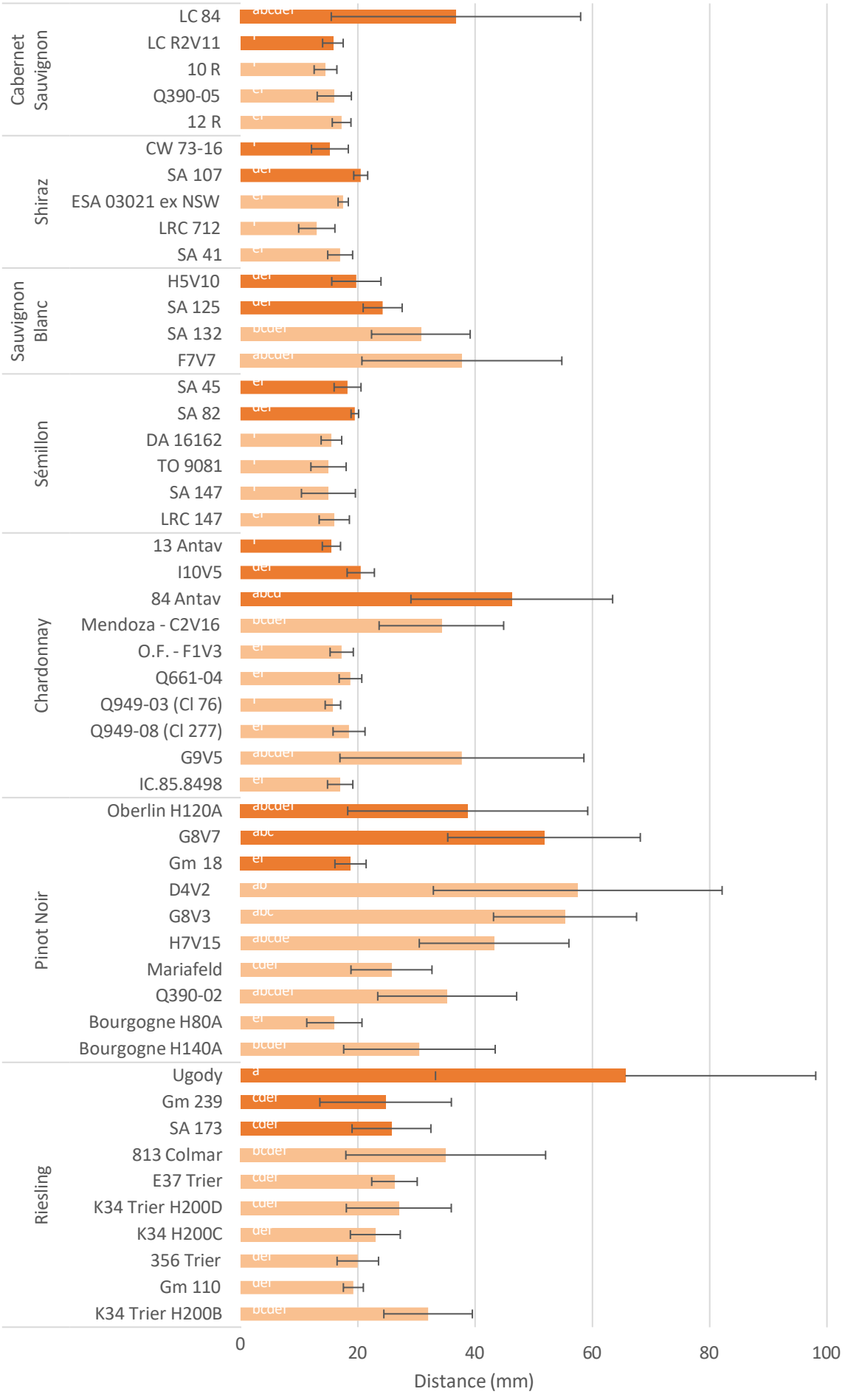


Figure 10.14. Lesion length six months after pruning detached canes (non-inoculated controls) of selected clones and cultivars from the clone collection at Nuriootpa Research Centre. Dark coloured columns represent clones with greatest dieback severity from visual assessments, and light columns the least. Columns with no letters in common are significantly different from each other (*P*<0.05) and bars represent

standard errors of the means.

smaller for clones with higher visual dieback assessments for cvs. Shiraz, Sauvignon Blanc and Chardonnay, with the other cultivars having no apparent relationship between visual assessment and cane diameter. There was no correlation between mean cane diameter and pathogen recovery distance (R^2<0.06) or lesion length (R^2<0.05) for any cultivars (data not shown).

Botryosphaeria dieback

In the BD DCA, *D. seriata* was recovered from canes of all cultivars, mean distances of which ranged between 14 and 24 mm (Cabernet Savignon), 16 and 22 mm (Shiraz), 17 and 46 mm (Sauvignon Blanc), 11 and 24 mm (Sémillon), 28 and 52 mm (Chardonnay), 16 and 41 mm (Pinot Noir) and 19 and 51 mm (Riesling) from the wound site over the six months incubation period (Figure 10.15). *D. seriata* was not recovered from non-inoculated canes (data not shown). There was large variation in the data, evidenced by the standard error bars, and no significant differences (P>0.05) were found between clones within any cultivars.

The range of mean lesion lengths of lesions in canes inoculated with *D. seriata* for each cultivar crosses over with the range for NICs (Table 10.2, Figures 10.14 and 10.15). The correlation between lesion lengths for canes inoculated with *D. seriata* and NICs was R^2=0.3 (data not shown). There was no significant difference (*P*>0.05) between clones of any cultivar for length of lesions when inoculated with *D. seriata* (Figure 10.15).

Pathogen recovery generally exceeded the lesion length by between 2 and 23 mm across all cultivars, except Pinot Noir, for which lesion length generally exceeded pathogen recovery by between 5 and 16 mm (Table 10.3). For 24 of the 50 clones, there was 5 mm or less difference between pathogen recovery and lesion length. There was a slight correlation between pathogen recovery distance and lesion length for all cultivars (R^2=0.4; data not shown).

There was no correlation between mean cane diameter and pathogen recovery distance (R^2<0.04) or lesion length (R^2<0.02) for any cultivars (data not shown).

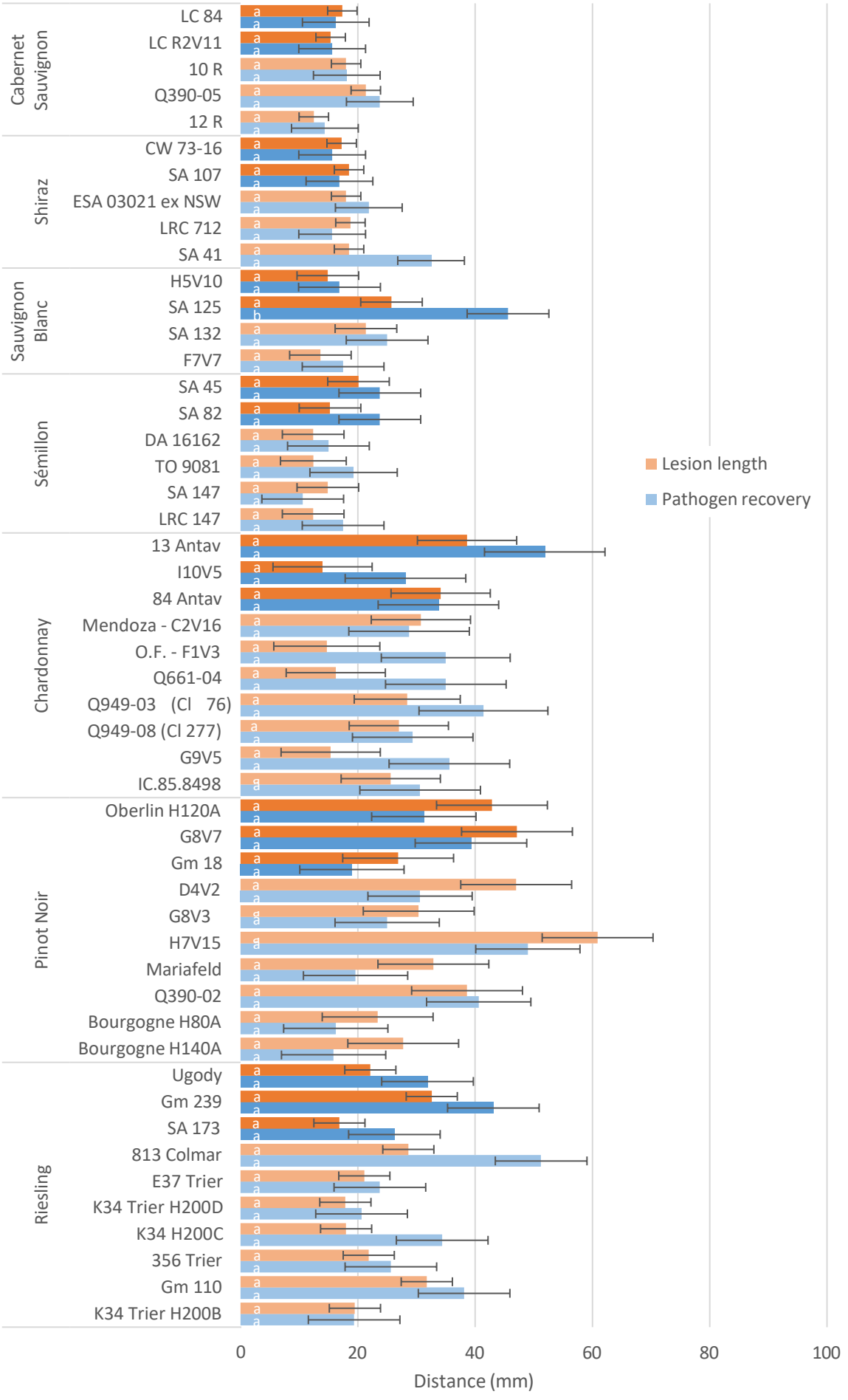


Figure 10.15. Recovery distance of *Diplodia seriata* (blue columns), lesion length (orange columns) 12 months after inoculation of pruning wounds on detached canes of selected clones and cultivars from the clone collection at Nuriootpa Research Centre. Dark coloured columns represent clones with greatest dieback severity from visual assessments, and light columns the least. Columns with no letters in common

are significantly different from each other (P<0.05) and bars represent standard errors of the means.

Table 10.3. Clones selected from the SARDI clonal collection located at the Nuriootpa Research Centre for greenhouse evaluation, including vine age, visual dieback severity rating, difference between either *Eutypa lata* or *Diplodia seriata* recovery and lesion length, and mean cane diameter. Clones in bold font represent those with the greatest dieback severity from visual assessments.

Cultivar	Clone	Vine age (y)	Dieback severity (%)	Recovery – lesion length (mm)		Cane diameter (mm)
				E. lata	*D. seriata*	
Cabernet Sauvignon	**LC 84**	**29**	**83**	**29**	**0**	**6.8 abc**
	LC R2V11	**29**	**70**	**41**	**6**	**6.6 abc**
	10 R	23	67	45	4	7.3 ab
	Q390-05	25	60	42	16	7.3 ab
	12 R	23	35	49	3	7.1 abc
Shiraz	**CW 73-16**	**33**	**80**	**17**	**3**	**6.0 c**
	SA 107	**na**	**80**	**19**	**23**	**6.2 bc**
	ESA 03021 ex NSW	29	67	6	9	6.8 abc
	LRC 712	24	57	25	11	7.6 a
	SA 41	na	45	27	10	7.8 a
Sauvignon Blanc	**H5V10**	**27**	**87**	**55**	**-12**	**5.0 d**
	SA 125	**na**	**80**	**53**	**-7**	**5.6 cd**
	SA 132	na	75	35	2	7.1 a
	F7V7	26	62	22	-13	6.1 abcd
Sémillon	**SA 45**	**na**	**63**	**48**	**-12**	**7.0 ab**
	SA 82	**na**	**50**	**39**	**-5**	**6.8 abc**
	DA 16162	32	15	20	-16	5.6 cd
	TO 9081	32	13	13	-8	5.5 d
	SA 147	28	8	22	-8	5.1 d
	LRC 147	28	7	12	-12	5.9 bcd
Chardonnay	**13 Antav**	**37**	**77**	**25**	**5**	**3.5 f**
	I10V5	**42**	**62**	**34**	**20**	**4.4 de**
	84 Antav	**37**	**57**	**38**	**2**	**3.8 ef**
	Mendoza - C2V16	48	27	25	13	4.9 cd
	O.F. - F1V3	48	27	40	19	4.0 ef
	Q661-04	25	13	54	20	5.0 bcd
	Q949-03 (CI 76)	25	7	65	-2	5.5 abc
	Q949-08 (CI 277)	25	5	44	0	5.8 a
	G9V5	26	4	35	14	5.6 ab
	IC.85.8498	21	2	33	13	5.5 abc
Pinot Noir	**Oberlin H120A**	**31**	**53**	**19**	**5**	**4.3 c**
	G8V7	**na**	**48**	**8**	**-4**	**5.0 b**
	Gm 18	**na**	**43**	**2**	**7**	**3.9 cd**
	D4V2	na	17	-62	3	3.4 d
	G8V3	na	17	-17	9	4.1 c
	H7V15	na	15	-8	4	5.1 b
	Mariafeld	na	13	0	4	4.0 cd
	Q390-02	na	13	1	4	6.3 a
	Bourgogne H80A	31	10	9	20	4.0 cd
	Bourgogne H140A	31	8	3	2	4.3 c
Riesling	**Ugody**	**na**	**53**	**36**	**14**	**5.9 ab**
	Gm 239	**na**	**43**	**43**	**-3**	**5.5 ab**
	SA 173	**na**	**40**	**30**	**4**	**5.1 b**
	813 Colmar	25	3	45	-2	5.3 b
	E37 Trier	25	3	62	-2	6.3 a
	K34 Trier H200D	25	3	25	2	5.8 ab
	K34 H200C	27	2	39	2	5.9 ab
	356 Trier	25	0	36	0	5.6 ab
	Gm 110	26	0	52	0	5.8 ab
	K34 Trier H200B	25	0	48	-1	5.5 ab

na = not available

10.3.3 Field rootstock evaluation

For the ED rootstock evaluation, *E. lata* was recovered from canes on all rootstocks and own rooted vines, mean distances of which ranged between 34 and 64 mm from the wound site 11 months after inoculation (Figure 10.16). There was no significant difference (P>0.05) between any of the rootstocks or own-rooted vines. Lesion length ranged between 38 and 83 mm, with Shiraz canes on rootstock Ramsey having significantly (P<0.05) greater lesion length than when on rootstock K51-40. Cane lesions on Shiraz vines on all other rootstocks were similar (P>0.05). There was no correlation between pathogen recovery and lesion length (R^2=0.0005).

For the BD rootstock evaluation, *D. seriata* was recovered from canes on all rootstocks and own rooted vines, mean distances of which ranged between 61 and 85 mm from the wound site 11 months after inoculation (Figure 10.17). There was no significant difference (P>0.05) between any of the rootstocks or own-rooted vines. Lesion length ranged between 37 and 61 mm, with no difference (P=0.05) between rootstocks. There was no correlation between pathogen recovery and lesion length (R^2=0.015).

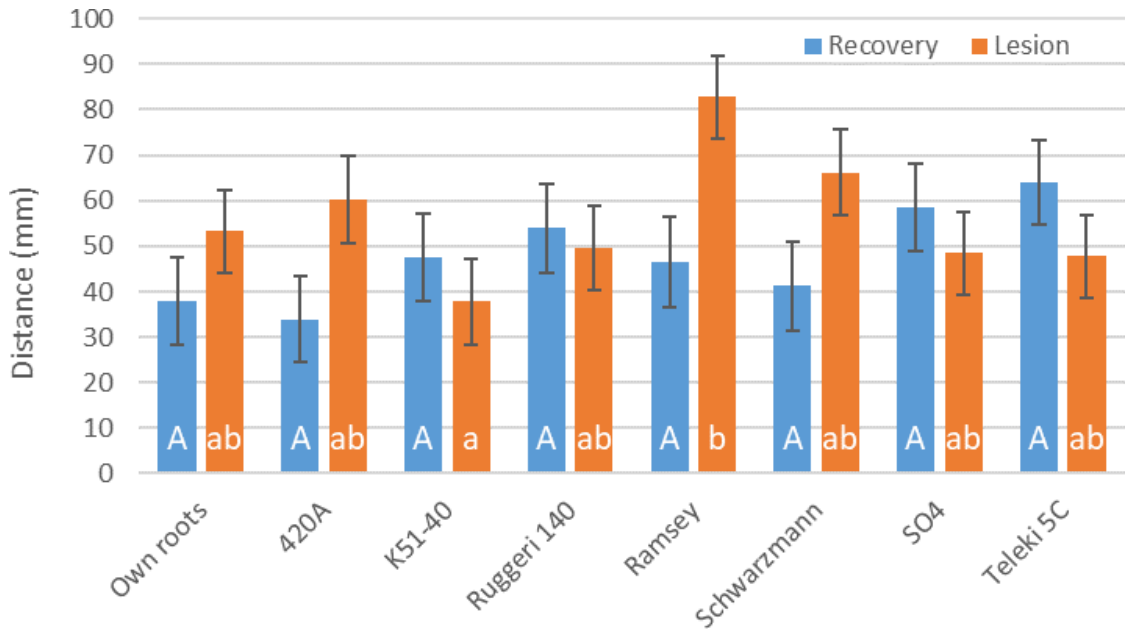


Figure 10.16. Recovery distance of *Eutypa lata* (blue columns), lesion length (orange columns) 12 months after inoculation of pruning wounds on canes of cv. Shiraz (clone BVRC12) on various rootstocks and on own roots in the Waite Campus vineyard in Urrbrae, SA. Columns with letters in common are not significantly different from each other (P>0.05) and bars represent standard errors of the means.

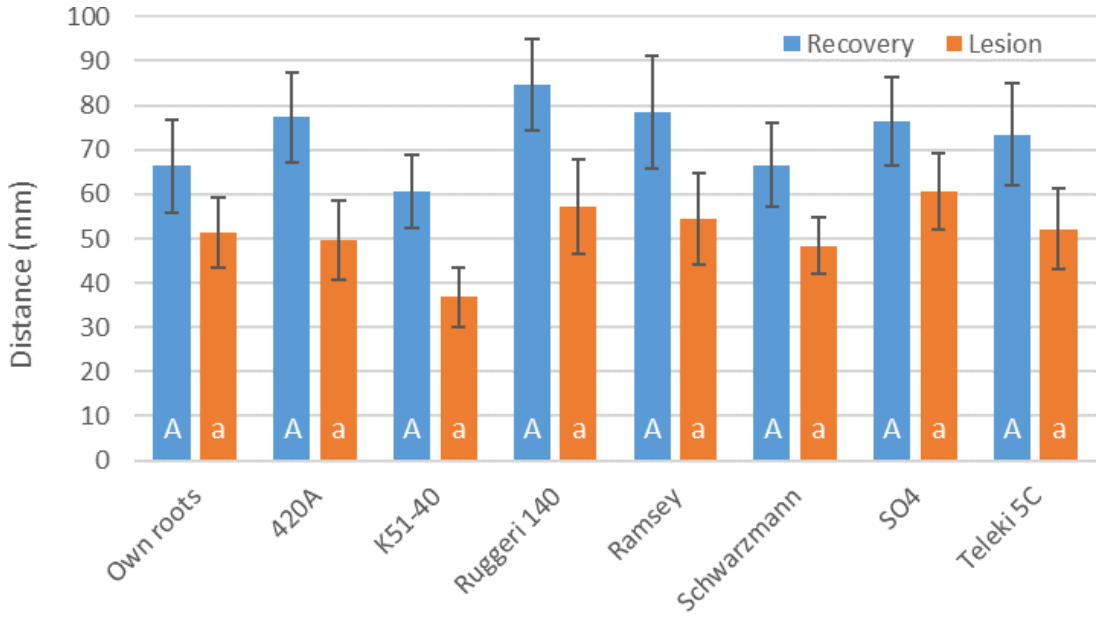


Figure 10.17. Recovery distance of *Diplodia seriata* (blue columns), lesion length (orange columns) 12 months after inoculation of pruning wounds on canes of cv. Shiraz (clone BVRC12) on various rootstocks and on own roots in the Waite Campus vineyard in Urrbrae, SA. Columns with letters in common are not significantly different from each other (*P*>0.05) and bars represent standard errors of the means.

10.4 Discussion

The visual assessment of symptoms in the SARDI germplasm clonal collection indicated that the severity of dieback varied considerably between the clones in the 21-48 year old vines. Although results were confounded by differences in vine age of the various clones and the lack of repetition in clonal plantings, they highlighted possible tolerance in some clones, based on dieback symptoms. The results showed that for the varieties Cabernet Sauvignon, Shiraz, Grenache and Sauvignon Blanc, previously reported to be susceptible to GTDs (Sosnowski *et al*. 2013a), most clones had severe dieback symptoms (above 50%), with only a few clones with potential of tolerance. However, for the varieties previously reported to be less susceptible, Pinot Noir, Chardonnay, Riesling and Semillon, most clones had less severe dieback symptoms (<50%) with larger variation than the susceptible varieties.

All vines were subjected to natural infection, as shown in previous studies on neighbouring vines at the Nuriootpa Research Centre, where up to 12% and 33% incidence of natural pruning wound infection has been reported for ED and BD dieback pathogens, respectively (Sosnowski *et al.* 2008, Pitt *et al*. 2012, Sosnowski *et al.* 2013). Furthermore, isolations from the germplasm cultivar collections, adjacent to the clonal collection, resulted in detection of both ED and BD pathogens from symptomatic vines (Sosnowski *et al*. 2017, 2022).

For the replicated trials in commercial vineyards, incidence of vines with foliar symptoms of ED was also recorded, although data were not analysed due to limited sample numbers with binary data. They did, however, provide confirmation of the presence of the ED pathogen, *E. lata*, the only species attributed to causing the characteristic foliar symptoms associated with this disease (Pitt *et al*. 2013). The incidence of foliar symptoms was not always consistent with the dieback severity. This may be due to the presence of other trunk pathogens, particularly those that cause BD, which do not express foliar symptoms. It has also been reported that foliar symptoms can vary from year to year (Sosnowski *et al*. 2007a) and do not reflect the severity of wood tissue necrosis (Sosnowski *et al*. 2007b).

There were no differences between Chardonnay clones in two of the replicated trials in commercial vineyards, but they did not include of any of the least symptomatic clones assessed in the germplasm collection. In the third commercial trial in the Clare Valley, although clones varied significantly, they only ranged between 40 and 50% dieback severity. There were differences observed between clones in the replicated Cabernet Sauvignon trial, whereas observations from the germplasm collection revealed little potential for tolerance. However, the clones showing the fewest symptoms in the commercial trial were not included in the germplasm collection. The commercial clonal Pinot Noir trial, which was planted in large, non-replicated blocks, revealed variation in dieback incidence. In the clonal germplasm collection, it was observed that Pinot Noir clones varied considerably in dieback severity, even where clones were of similar age. There were no replicated trials for Riesling, Sauvignon Blanc or Semillon clones, but observations from the germplasm collection suggest there may be some potential candidates for tolerance. Based on evidence from the germplasm collection, there is little potential for identifying tolerance from the available clones of Shiraz and Grenache.

Evaluation of a selection of clones in replicated experiments using DCAs has provided no evidence for any tolerance against *E. lata* or *D. seriata* infection amongst them. There was large variation in the distance that pathogens were recovered within clones, which made it impossible to distinguish between the mean distances for each cultivar. Furthermore, lesion lengths did not differ between clones of the same cultivar, and natural dieback, as represented by the NICs, was generally similar to that on canes inoculated with pathogens. Therefore, it is most likely that any differences in dieback observed on vines in the field may be due to other factors such as vine age or variation in the ability of clones to tolerate other causes of dieback, such as water stress or poor soil conditions. Traditionally, clonal selection is a process of selecting material from desirable vines (McCarthy 1988, Nicholas 1994), but this process appears not to have influenced the ability of clones to tolerate grapevine trunk pathogens.

Assessment of rootstock trials revealed variation of dieback symptoms between vines planted on rootstocks and on own roots. In the Chardonnay trials, own rooted vines had less severe dieback than vines planted on most rootstocks. Cabernet Sauvignon vines varied in dieback severity on rootstocks, with own rooted vines among those with the least severe dieback symptoms. On the other hand, in two of three trials, Shiraz vines on own roots were recorded with more severe dieback symptoms than those on most rootstocks.

Like the clone evaluations, rootstocks did not appear to confer tolerance on Shiraz scion canes inoculated with *E. lata* or *D. seriata* in a vineyard trial. Again, differences in dieback between vines on different rootstocks or own roots that were observed in the vineyard may be due to other factors such as their adaption to water stress, different soil conditions or soilborne pathogens such as nematodes (Whiting 2004).

In these evaluations there was no correlation between pathogen recovery distance and lesion length, and in the case of the DCA experiments, inoculated canes had similar lesion lengths to natural dieback in NICs, which supports previous reports that lesion length alone is not a reliable measure of disease (Sosnowski *et al.* 2007a, 2021, 2022). Also, *E. lata* was recovered at a greater distance from the inoculation point than *D. seriata* in DCAs, compared to similar distances between the pathogens recorded in the vineyard experiment. This contradicts reports of Sosnowski *et al.* (2022) where *E. lata* was more pathogenic than *D. seriata* in vineyards trials, when compared with DCAs. However, the DCA in the current study was incubated for six months, compared with only one month in the DCA of Sosnowski *et al.* (2022), but, nevertheless, *D. seriata* was recovered slightly further from the inoculation point than *E. lata* in the vineyard trial.

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11 NURSERY PROPAGATION

11.1 Characterisation of the endophytic microbiome in nursery propagation material

11.1.1 Introduction

The plant endophytic microbiota is the community of microorganisms (bacteria, fungi, viruses) that naturally resides within plants (Hardoim *et al*. 2015). These microbiota can be harmless, beneficial or pathogenic to their plant host (Hardoim *et al*. 2015; Pinto *et al.* 2014; Rybakova *et al.*, 2015; Brader *et al.*, 2017). The diversity of microbiota in an individual plant can be influenced by variety, age and climatic factors, as well as management practices such as organic vs. conventional methods (Pinto *et al.* 2014; Vitulo *et al.* 2019). A well-balanced and diverse population of these microbiota plays a very important role in plant health and adaptation to changing environmental conditions by increasing plant fitness. Furthermore, the beneficial microorganisms may also protect plants against harmful organisms by keeping their population low, while at the same time reinforcing the natural plant defence mechanisms (Pinto *et al*. 2014; Rybakova *et al*. 2015). Occasionally however, plants become exposed to extreme stress factors including nutrient deficiencies, heat or water stress or mechanical damage. These stressors may cause an imbalance in the microbiota by favouring harmful over beneficial organisms, resulting in poor plant growth, disease and other disorders.

Studies from overseas showed that fungi associated with Petri disease, black foot and Botryosphaeria dieback (BD) were present in propagation cuttings and young dormant vines (Halleen *et al.* 2003; Gramaje *et al.* 2009, Gramaje and Armengol 2011; Bleach *et al.* 2013, Billones-Baaijens 2013). These studies indicate a likely link between the presence of these pathogens in nursery vines and the low survival rates of young vines in new vineyards. However, the prevalence and distribution of fungal pathogens in grapevine nurseries in Australia remain unclear and, to our knowledge, there have been no comprehensive surveys of nursery plant materials carried out to date (Billones-Baaijens and Savocchia 2018). To mitigate the negative effects of these pathogens in young vines, hot water treatment (HWT) of different temperatures and duration has been widely used for disinfecting propagation materials and grafted vines in nurseries around the world, including Australia (Fourie and Halleen 2004b; Waite and May 2005; Halleen *et al.* 2007; Gramaje *et al.* 2009; Bleach *et al.* 2013; Gramaje and Armengol 2011). While many nurseries recognise HWT as a management option in reducing some of the GTD infections in young vines, serious concerns have been raised due to issues with poor growth and vine deaths that were associated with this practice (Gramaje and Armengol 2011; Billones *et al.* 2014; Gramaje *et al.* 2018). The current Australian Standard for Grapevine Propagation Material (Standards Australia 2013) recommends HWT at 50°C for 30 min as an internal treatment to reduce crown gall and trunk disease. The Standard further recommends the HWT of 55°C for 5 min for the external treatment of propagation material to reduce surface fungi, bacteria, phylloxera and nematodes.

Healthy and productive vineyards are key to a sustainable wine industry. However, a changing climate and the emergence of new pests and diseases are making vineyard management more challenging in recent years. Good quality and disease-free planting materials will most likely withstand extreme vineyard conditions better than poor quality materials. Thus, investigating the microbiota associated with grapevine planting materials may help nurseries to better understand the overall health status of their planting materials. This may provide opportunities to manipulate the microbiota and to improve the fitness of the vines, so they are able to better adapt to a changing environment.

In recent years, the study of the different microbiota has become more common with the development of Next Generation Sequencing (NGS). This DNA-based technology allows rapid and accurate identification of all the genes (microbiome) of different microbiota of plants including grapevines (Pinto *et al*. 2014; Vitulo *et al*. 2019). The first objective of this study was to characterise the endophytic microorganisms present in grapevine nursery planting materials from different grapevine nurseries. This study identified the most abundant and rare endophytic microorganisms, and investigated their associations with different nurseries, varieties, plant type (grafted vs. own-rooted vines) and parts of the plant (scion, rootstock, roots). This information will help in understanding the dynamics of these endophytic microorganisms that may play a role in the health and fitness of planting material and young vines in vineyards. The second objective of this study was to investigate the effects of HWT on microbial communities in young vines, aimed at clarifying the benefits of this practice in the health and fitness of young vines.

11.1.2 Methods

11.1.2.1 Sample collection from different nurseries

Samples were collected from four grapevine nurseries in Australia in September-October 2019. Each nursery was requested to send five to ten samples of each plant material type, which comprised dormant, apparently healthy grafted plants and own-rooted vines (Table 11.1). Samples were sent by courier to the NWGIC plant pathology laboratory and stored at 4°C until processed within 1-2 weeks of arrival. Full confidentiality was maintained regarding nurseries contributing materials to the study.

Table 11.1. Grafted and own-rooted vines collected from different grapevine nurseries across Australia and used for microbial profiling.

Nursery	Number of grafted vines				Number of own-rooted vines	
	Chardonnay x Paulsen	Chardonnay x Ramsey	Chardonnay x 101-14	Shiraz x Paulsen	Chardonnay	Shiraz
1	4	-	-	4	-	5
2	-	4	-	4	-	-
3	-	-	-	-	-	5
4	-	-	5	5	5	5
TOTAL	4	4	5	13	5	15

11.1.2.2 Sample processing and DNA extractions

All plants were washed thoroughly with tap water to remove any debris and soil and air-dried for ~1 h. All bark was removed before each plant was cut to separate the scion, rootstock trunk and roots in the case of the grafted vines (Figure 11.1a), and trunk and roots in the case of own-rooted vines (Figure 11.1b). Each tissue type was further cut into ~1 cm sections before surface-sterilising with 70% ethanol for 2 min, rinsed twice with sterile deionised water (SDW) for 1 min and air-dried inside the laminar flow cabinet for ~30 minutes. All samples were homogenised using a sterile coffee grinder before transferring sub-samples into 15 mL tubes and storing at -80°C for 7-14 days until freeze-drying. All wood samples were freeze-dried (Christ, John Morris Scientific, U.S.A.) for 36 h, and dried samples were pulverised at 20 Hz for 2 min 30 s using 10 mL grinding jars attached to a TissueLyser II (Qiagen, U.S.A.). The ground wood (100 mg) was transferred into a sterile 2 mL tube for the DNA extraction using the DNEasy Plant Pro Kit following the manufacturer's protocol (Qiagen, USA). A total of 118 DNA samples were extracted and submitted to the Australian Genome Research Facilities (AGRF), Melbourne for NGS analysis.

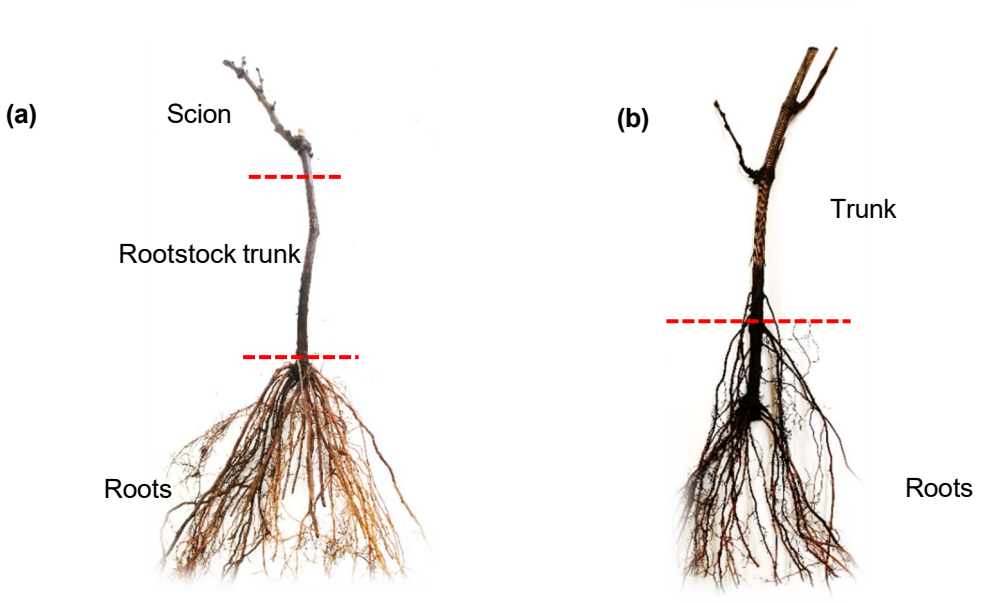


Figure 11.1. Nursery plant materials showing the positions of the tissue samples used for DNA extractions and next generation sequencing: a) grafted vine and; b) own-rooted vine.

11.1.2.3 Effects of hot water treatment on plant microbiome

In this study, the HWT protocol at 54°C for 5 min adopted from the current Australian Standard for external treatment of grapevine material (Australian Standard 5588-2013), with minor modification, was used to investigate the effects of 'brief' hot water treatment on the vine microbiome. This protocol is generally used as a biosecurity measure for transporting grapevine plant materials across different states in Australia (Nick Dry, pers com July 2019). In September 2020, dormant one-year old vines that had not been subjected to HWT were procured from Nursery 2 and transported to Wagga Wagga, NSW at ambient temperature (Table 11.2). Upon arrival, all vines were stored in a cool room (4°C) for one week until processed. Vines were removed from the cool room and placed at room temperature (20°C) overnight. All vines were rehydrated by soaking in tap water for 30 min at room temperature prior to hot water treatment. Five vines for each plant type and variety were randomly assigned to different treatments: 1) hot water treatment (HWT); or 2) non-HWT (control). For HWT, five vines representing five replicates per plant type/variety were emersed for 5 min in a temperature-controlled hot water bath at 54°C and cooled by 30 min immersion in plastic tubs containing deionised water (~20°C). To prevent cross contamination, four separate hot water and cooling baths were used for each plant type and variety combination. Negative control vines were soaked in deionised water (~20°C) for 30 min. Canes for all treatments were packed separately in perforated bags and stored at 12-15°C for a week to simulate the storage conditions and approximate time for transporting vines for delivery to customers.

After post-HWT storage, each plant was cut to separate the scion, rootstock trunk and roots, in the case of grafted vines (Figure 11.1a) and trunk and roots, in the case of own-rooted vines (Figure 11.1b). The bark from each tissue type was also removed and placed into 15 mL tubes. DNA extraction from all woody tissues was carried out using the Qiagen Plant Pro Kit (Qiagen, USA) as described in the first nursery sampling section. For the bark, DNA extraction was carried out using the PowerSoil DNA Isolation Kit (Qiagen, USA) following the protocol developed by Vitulo *et al.* (2019).

Table 11.2. Number of grafted vines and own-rooted vines collected from Nursery 2 and used to investigate the effects of hot water treatment or no hot water treatment on the plant microbiome.

Plant type	Variety (x Rootstock)	Hot water treatment (54°C – 5 min)	No hot water treatment
Grafted	Shiraz x Paulsen	5	5
	Sauvignon blanc x Paulsen	5	5
Own-rooted vines	Shiraz	5	5
	Sauvignon blanc	5	5

11.1.2.4 Next generation sequencing and data analysis

All PCR amplifications and sequencing were performed by the AGRF using the primers in Table 11.3. The thermal cycling conditions were as follows: initial denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 60°C for 10 s and 30 s at 72°C and a final extension of 72°C for 5 minutes. All samples were sequenced using an Illumina MiSeq (San Diego, CA, USA) with a V3, 600 cycle kit (2 x 300 base pairs paired-end).

Table 11.3. Primers used for PCR amplifications of bacteria and fungi.

Target	Primers	Sequence
ITS	ITS1F	5'CTTGGTCATTTAGAGGAAGTAA3'
	ITS2	5'GCTGCGTTCTTCATCGATGC3'
16S V3-V4	341F	5'CCTAYGGGRBGCASCAG3'
	806R	5'GGACTACNNGGGTATCTAAT3'

After high-throughput sequencing, downstream bioinformatics analysis was performed by AGRF. Sequences were quality filtered, and full-length duplicate sequences were removed and sorted by abundance using USEARCH tools. Singletons or unique reads in the data set were discarded. Sequences were clustered to filter out chimeric sequences using the 'rdp gold' database as a reference. The metagenomic data were analysed using state-of-the-art pipeline and databases for the analysis of the microbiome dataset. The final output was an amplicon sequence variant (ASV) table, which records the number of times each exact amplicon sequence variant was observed in each sample. The number of reads in each ASVs was obtained by mapping the reads back with a minimum identity of 97%. Taxonomy was assigned using QIIME2 (Bolyen *et al.* 2019)

11.1.3 Results

11.1.3.1 Nursery microbial profiling

Metagenomic data for microbial diversity profiling were generated from the 118 DNA samples collected from the four nurseries, while two DNA samples failed the quality control and were excluded from the analysis. Only those taxa that represent more than 1% of the bacterial and fungal ASVs were included in the analysis. Chloroplast and mitochondrial sequences or ASVs with unidentified taxa were also filtered out. Percent relative abundance of the bacterial and fungal taxa was calculated and the composition and diversity of the microbial community in different varieties and tissue types from different nurseries were compared and characterised.

The comparisons of microbial communities present from different nursery materials were carried out at family and genus levels for the bacteria and fungi, respectively. For bacterial communities, the bacterial populations at family level were ranked and the top 10 populations were used to compare diversity between different variables. For fungal communities, genera were assigned into four groups: (1) fungi associated with young vine decline (YVD); (2) bunch rot pathogens; (3) fungi with known biocontrol potential (beneficial); while the remaining genera that do not belong in the above categories were classified as others (4). The relative abundance and diversity of the fungal groups were compared, to determine differences between variables.

Fungal communities

Metagenomic data from the 118 samples obtained from the four nurseries identified a total of 298 fungal genera. The fungal communities were dominated by Ascomycete fungi that accounted for 59% of the total genera, followed by Basidiomycetes at 30%, while the proportion of mycorrhizal fungi, Glomeromycetes, was less than 1%. Of the 298 genera, 273 were considered environmental fungi of unknown importance to viticulture while a small number of genera were identified as beneficial (n=8), young vine decline pathogens (n=13) and bunch rot pathogens (n=4).

The relative abundance of fungal groups detected across different plant types, nurseries, varieties and tissues are shown in Figure 11.2. Overall fungal populations varied greatly between nurseries, varieties and tissue-type while fungal populations were similar between grafted and own-rooted vines (Figure 11.2a). Nursery 3 had the highest overall fungal populations, compared to the other three nurseries (Figure 11.2b), and a high proportion of young vine decline (YVD) pathogens were also detected from Nursery 2 and 3 vines. Beneficial fungi were detected in small numbers across the different nurseries. Ramsey had the highest fungal populations among rootstocks, while fungal populations were similar for Shiraz and Chardonnay (Figure 11.2c). A high proportion of YVD pathogens were further detected from Ramsey that was collected from Nursery 2.

Fungal populations in grafted vines varied between tissue types, with higher fungal populations for above ground tissues compared to roots (Figure 11.2d). In contrast, fungal populations for own-rooted vines were higher in the roots than the above ground tissues. Young vine decline pathogens were also detected in small proportions for all tissue types, with the lowest proportion detected from the scion of grafted vines.

The relative abundance of fungi associated with YVD is presented in Figure 11.3. Petri disease pathogens such as *Cadophora luteo-olivacea*, *Phaeoacremonium iranianum* and *Phaeomoniella chlamydospora* were the most abundant among YVD pathogens and were detected in all tissue types, with higher abundance in grafted vine roots and trunks than in own-root vines (Figure 11.3a). The black foot pathogen, *Ilyonectria macrodidyma*, was also detected, with highest proportion in the roots of the own-rooted vines and lesser proportion in the roots of grafted vines. Black foot pathogens were not detected in the scion of the grafted vines. The BD pathogens *Lasiodiplodia* and *Macrophomina* were also detected, but in significantly lower abundance than the Petri disease pathogens.

Beneficial fungi were also detected in different tissue types, with *Trichoderma* species being the most abundant genera in the scion and rootling trunks (Figure 11.3b). Mycorrhizal fungi (Glomeromycetes) were also detected in the roots, with the highest proportion in own-rooted vines. Mycorrhiza are fungi known to form a symbiotic relationship with roots of different plant species.

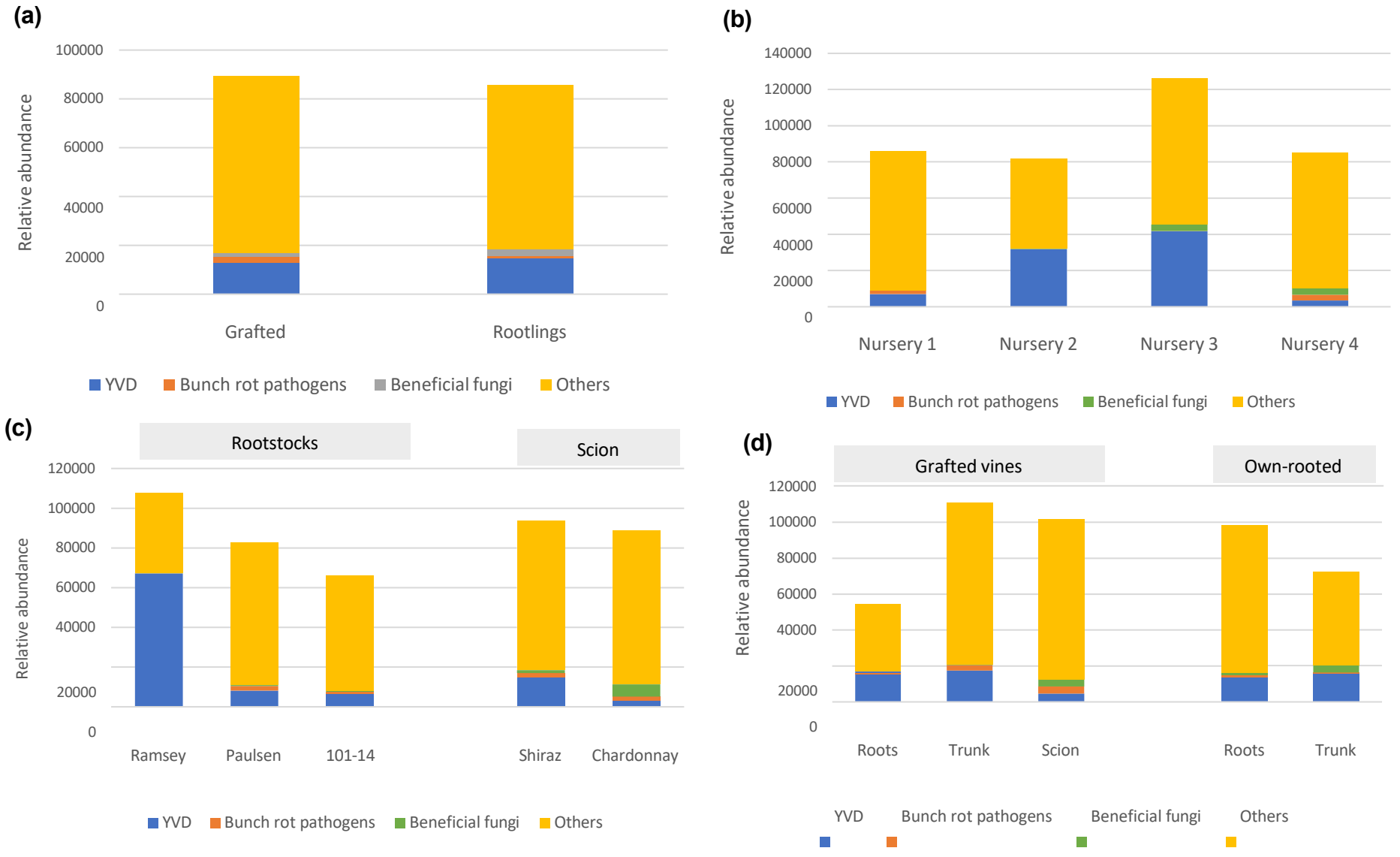


Figure 11.2. Relative abundance of fungal groups between (a) plant type; (b) nurseries; (c) varieties; and (d) tissue types. Different colours within a bar represent the

fungal communities that were assigned into four groups: fungi associated with young vine decline (YVD); bunch rot pathogens; fungi with known biocontrol potential (beneficial); fungi outside of these categories (others).

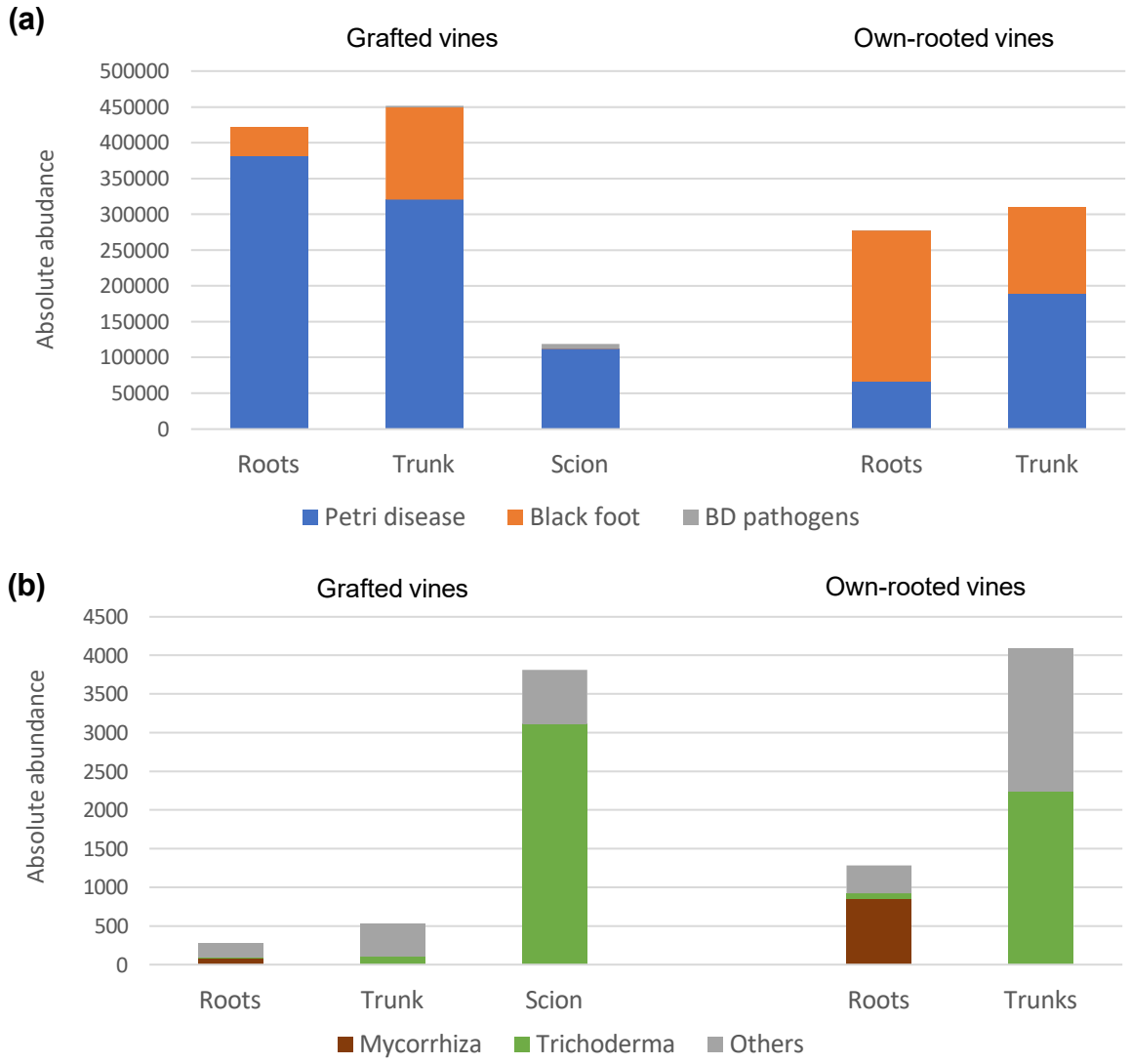


Figure 11.3. Abundance and distribution of young vine decline pathogens (a) and beneficial fungi (b) in different tissue types of grafted and own-rooted vines.

Bacterial communities

For the bacteria, a total of 198 communities were identified. Among the top 10 most abundant families (Figure 11.4), the Pseudomonadaceae and Burkholderiaceae ranked the highest at 21% and 20%, respectively. Nursery was a major factor in the variation, which was driven by the high abundance of Burkholderiaceae in Nursery 3, while the Pseudomonaceae dominated Nursery 2 (Figure 11.4a). Tissue type was also a factor in bacterial diversity, which was driven by the abundance of Burkholderiaceae and Micromonosporaceae in own-rooted vine roots (Figure 11.4b). A high proportion of Pseudomonadaceae were also detected in roots. Many species belonging to the Pseudomonadaceae and Burkholderiaceae families are known to be plant-growth-promoting rhizobacteria, although a few species are known to be animal or plant pathogens. The soil bacteria family Rhizobiaceae, which is reported in nitrogen fixation in leguminous plants, ranked fourth in abundance. The remaining bacterial families identified were generally considered as environmental bacteria, but their importance to viticulture is unknown.

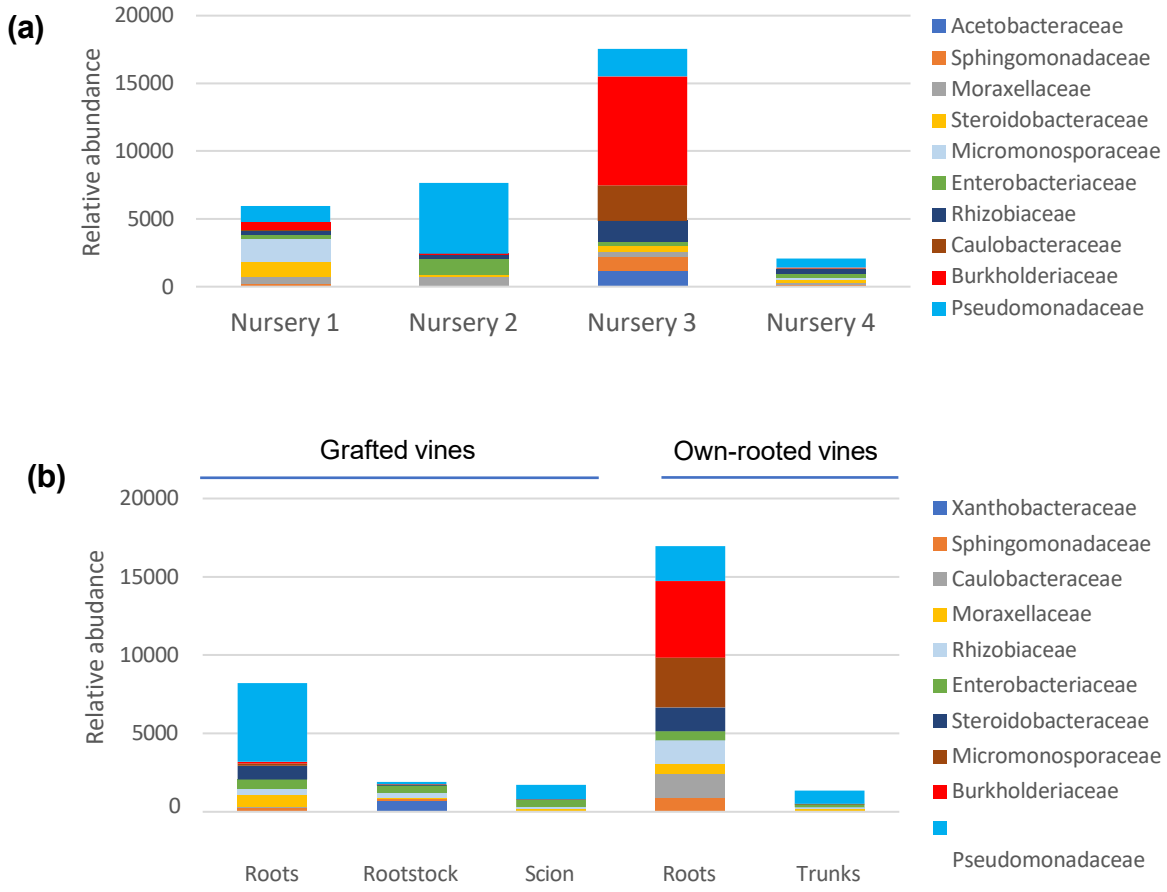


Figure 11.4. Relative abundance of the top 10 most abundant bacterial family in (1) nurseries and tissue types of grafted and own-rooted vines (2).

The overall relative diversity and abundance between bacterial communities at the family level varied between nurseries, plant type, variety and tissue-type, as illustrated in the heat maps (Figure 11.5). Among nurseries, Nursery 3 had the highest number of bacterial communities followed by Nursery 1, while bacterial communities from Nurseries 2 and 4 were relatively lower (Figure 11.5a). For plant type, own-rooted vines had a higher proportion of bacterial communities than grafted vines (Figure 11.5b). For varieties, Paulsen had the highest proportion of bacterial communities among rootstocks while Shiraz had the highest proportion of bacterial communities among the *V. vinifera* varieties (Figure 1.5c). For tissue types, the roots had the highest proportion of bacterial communities, with a greater abundance from the roots of own-rooted vines, compared to grafted vine roots (Figure 11.5d). For

above-ground tissues, the proportion of bacterial communities was similar for the grafted and own-rooted vine trunks while bacterial communities were relatively lower for the scion tissues.

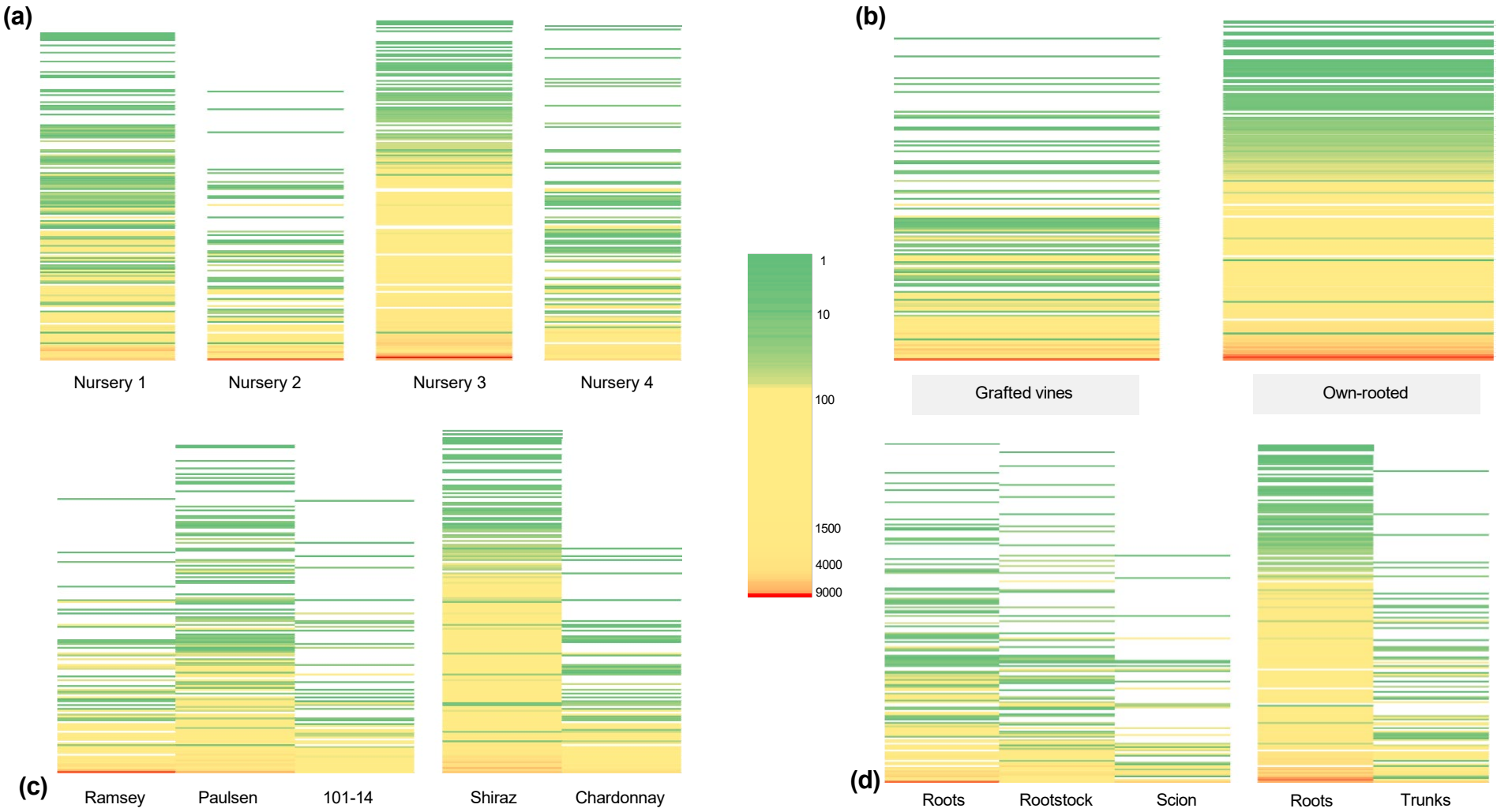


Figure 11.5. Heatmap of the bacterial populations from grapevine plant materials showing the differences in diversity between nurseries (a); plant types (b); varieties (c); and tissue types (d) The lines of different colours for each bar represent the different bacterial families and their relative abundance as indicated in the colour scale. White space represents bacterial families that were absent within each factor.

11.1.3.2 Effects of Hot Water Treatment (HWT)

Fungal communities

A total of 254 fungal ASVs were obtained from the 140 DNA samples analysed. However, only the top 88 ASVs identified at the genus level with the most abundant reads were included in the analysis. The fungal communities were dominated by environmental fungi (69 genera) of unknown importance to viticulture (referred here as others), while a small proportion were identified as young vine decline pathogens (8 genera), beneficial fungi (7 genera), and bunch rot pathogens (4 genera).

Overall results showed HWT at 54°C for 5 min had no significant effect on the fungal communities in dormant vines (Figure 11.6). The relative abundance for each fungal group including YVD pathogens was similar between plant types and varieties, suggesting the fungal communities in young vines can tolerate the HWT at 54°C for 5 min.

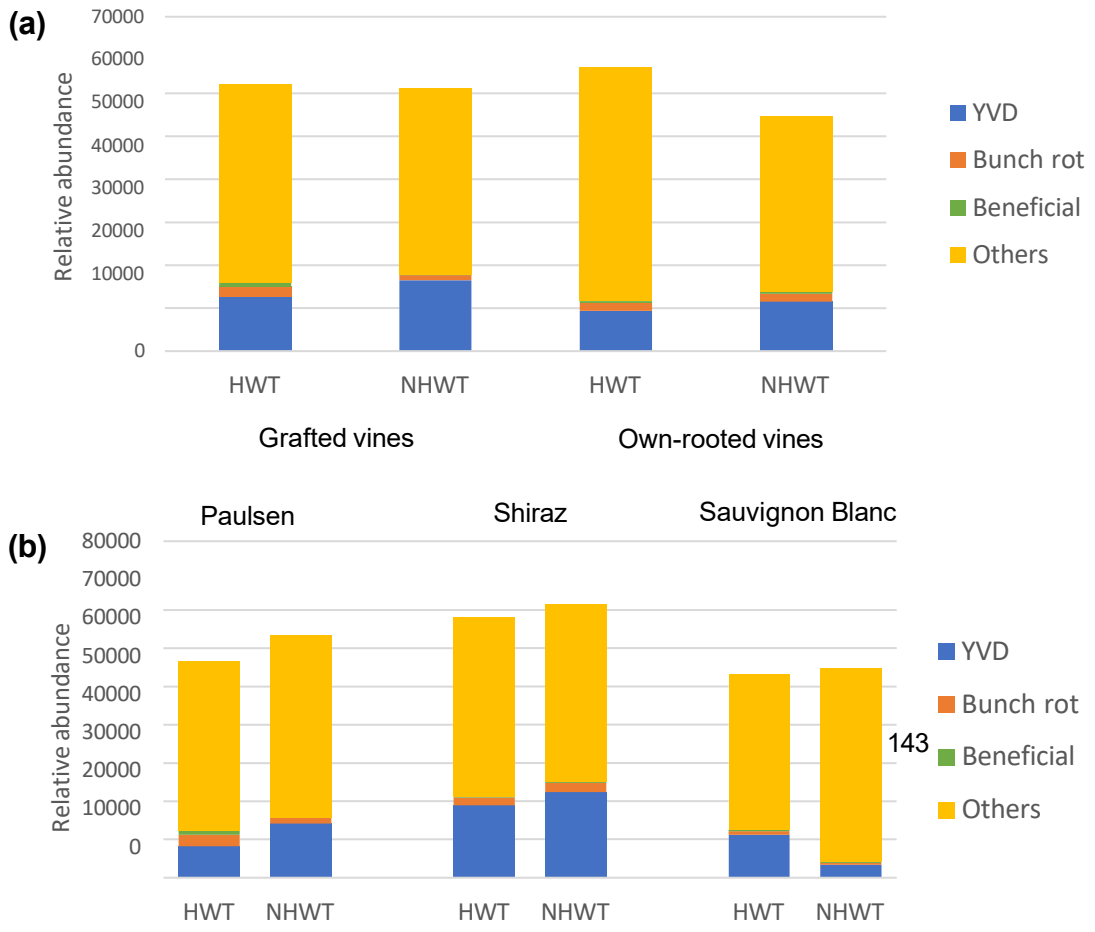


Figure 11.6. Effects of hot water treatment (HWT) at 54°C for 5 min on the relative abundance of different fungal groups on (a) plant types and (b) varieties. Colours within bars represent the fungal communities that were assigned into four groups: fungi associated with young vine decline (YVD); bunch rot pathogens; fungi with known biocontrol potential (beneficial); fungi outside of these categories (others). NHWT – no hot water treatment control.

Bacterial communities

A total of 155 bacterial communities were identified from 140 DNA samples analysed. Among the top 10 most abundant families (Figure 11.7), the Pseudomonadaceae ranked the highest at 27%, followed by Rhizobiaceae (13%), Spingomonadaceae (11%), Bacillaceae (10%) and Microbacteriaceae (10%), while the proportion of the five remaining families ranged from 5 - 8%.

For grafted vines, HWT was a factor in the variation of bacterial populations, which was primarily driven by a significant increase in abundance of Bacillaceae for vines treated with HWT, compared to the non-HWT control vines (Figure 11.7a). For own-rooted vines, HWT was also a factor in the variation of bacterial populations, which was primarily driven by a significant decrease in abundance of Pseudomonadaceae and a slight increase of Bacillaceae for HWT-treated vines, compared to the non-HWT control vines. For varieties, HWT was also a factor in the variation of bacterial populations, which was driven by an increase in abundance of Bacillaceae in Paulsen and a decrease in abundance of Pseudomonadaceae in Shiraz treated with HWT, compared to their corresponding non-HWT controls (Figure 1.7b). A slight increase in abundance of Bacillaceae for Shiraz and Sauvignon blanc was further observed for these varieties treated with HWT, while the abundance of Pseudomonadaceae for Paulsen and Sauvignon was similar for all treatments.

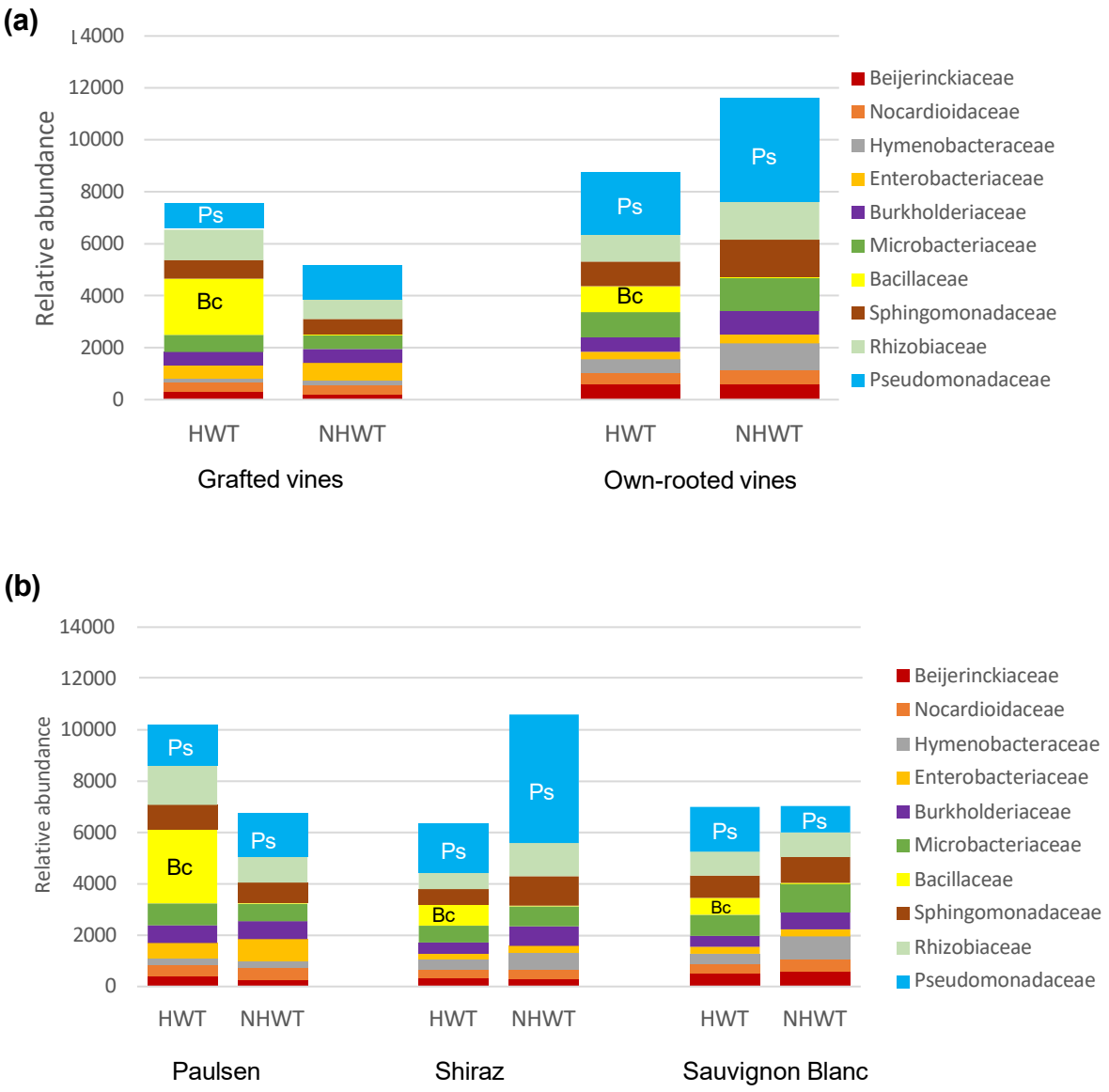


Figure 11.7. Effects of hot-water treatment (HWT) at 54°C for 5 min on the relative abundance of bacterial families in different (a) plant types and (b) varieties. Different colours within bars represent the 10 most abundant bacterial families. Ps – Pseudomonadaceae, Bc - Bacillaceae . NHWT – non-HWT control.

11.1.4 Discussion

11.1.4.1 Nursery microbial profiling

This study was carried out to investigate the diversity and relative abundance of endophytic microorganisms within nursery vines from four different Australian nurseries using high throughput amplicon sequencing (HTAS) NGS. It also explored the microbial diversity of the different biological factors including nursery source, varieties, plant types and tissues on the abundance and distribution of these endophytic microorganisms within the vine. Understanding the diversity of endophytic microbiota associated with grapevine plant materials may provide information on the overall health status of young vines, to assist nurseries in understanding the roles of these endophytic organisms in the quality and establishment of their vines in the vineyard.

This study showed microbial communities greatly varied between nurseries, varieties and tissue type. Nursery 3 had the highest overall microbial populations, compared to the other three nurseries, even though it provided only five own-rooted vines (cv. Shiraz). Ramsey had the highest microbial population among rootstocks while microbial populations were similar for Shiraz and Chardonnay. Microbial populations were also higher in the roots than the above ground tissues. These results are consistent with the study by Lade *et al.* (2022a) showing that nurseries, followed by rootstock and variety, are the most significant factors that influence the fungal community in young vines in Spanish nurseries.

In the case of fungi, 92% of the fungal genera identified are considered to be environmental fungi and their importance to viticulture is still unknown, while the remaining genera were associated with YVD, bunch rots or fungal species with biocontrol potential, and were classified as beneficial. For fungal genera associated with YVD, the Petri disease pathogens *Cadophora luteo-olivacaea, Phaeoacremonium* spp. and *P. chlamydopora* and black foot pathogen *Ilyonectria* were the most abundant but their distribution differed, with *C. luteo-olivacea* and *Phaeoacremonium* being abundant in all tissue types, *Ph. chlamydospora* being most abundant in the rootstock trunk, while *Ilyonectria* sp. was more abundant in the roots of own-rooted vines. The relative abundance and ubiquitous nature of these YVD pathogens were also in line with other microbial diversity studies in other grapevine nurseries (Eichmeier *et al.* 2018; Gramaje *et al.* 2022; Lade *et al.* 2022a, 2022b). The BD pathogens *Diplodia spp.* and *Lasiodiplodia* spp. were also detected in very small numbers (~1% of the total YVD abundance). The very low detection of BD pathogens in this study is in contrast with the studies conducted in New Zealand (Billones-Baaijens et al. 2013), South Africa (Fourier and Halleen 2004) and Spain (Aroca *et al.* 2012) where BD pathogens were commonly detected in different nursery plant materials.

Some beneficial fungi were also present in the nursery vines, although their relative abundance was lower than that of other fungal groups. *Trichoderma* spp. were predominant, and mostly associated with above-ground tissues. Many *Trichoderma* species are well known biocontrol agents against trunk disease pathogens (John *et al.* 2008; Pertot 2016). The Glomeromycetes, also known as arbuscular mycorrhizal fungi (AMF), were detected primarily in the roots of own-rooted vines, suggesting the *V. vinifera* roots maybe more efficient in recruiting these root-associated fungi, compared to rootstock species. A study by Goddard *et al.* (2021) suggested that the AMF colonisation of grapevine roots may provide better resistance to various pathogens. Carbone *et al.* (2021) also found AMF were higher in drought-stressed grapevine roots, suggesting that they may contribute to plant survival during extreme environmental conditions. Future studies to investigate the functionality of these AMFs in vine roots in Australia vineyards may lead to developing recommendations on varieties best suited for different climatic regions. Other species such as *Aureobasidum* sp., *Chaetomium* and *Clonostachys spp.* were also detected, suggesting young vines were harbouring a wide variety of beneficial fungi.

In the case of bacteria, the most predominant families were Pseudomonadaceae and Burkholderiaceae. Many *Pseudomonas* and *Burkholderia* species are well known for their biocontrol potential. For example, a *Pseudomonas poae* strain (BCA17) isolated from grapevine wood was found to be antagonistic against GTDs, resulting in a reduction of *N. luteum* infections in potted vines by 80% (Niem *et al.* 2020a, 2020b). The Gram-negative genus *Burkholderia* are also known nitrogen-fixing bacteria that are diverse in soils (Hall *et al.* 2015). For example, *Burkholderia phytofirmans* PsJN, is a highly efficient plant beneficial endophytic bacterium that promotes growth in a wide variety of plants including grapevine (Miotto-Vilanova *et al.* 2016). This bacterium also induces plant resistance against abiotic and biotic stresses. Since the HTAS method used in this study can only identify bacterial communities at a family level, it is important to clarify the identity of these highly abundant bacteria, since some are also known animal and/or human pathogens. It is important to note that the abundance of

Burkholderiaceae was directly linked to Nursery 3, suggesting that their abundance may be influenced by biogeographic factors and nursery practices. Vitulo *et al.* (2019) found that geographic patterns and agronomic management greatly influenced bacterial diversity in *V. vinifera* bark and grape berries.

YVD pathogens such as *C. luteo-olivacea*, *Phaeoacremonium* and *Ilyonectria* were found to be ubiquitous in all grapevine nurseries surveyed. However, the role of *Cadophora* species in the grapevine trunk diseases observed in Australia vineyards remains unknown to date. Small-scale studies on Petri disease (Edwards and Pascoe 2004) and black foot (Weckert *et al.* 2013) were conducted in previous years, however, their full impact in Australian vineyards also remains unclear. The importance of *Cadophora* species has also been questioned in recent years, since it has been isolated from both asymptomatic (Halleen *et al.* 2007; Casieri *et al.* 2009) and symptomatic vines from studies conducted overseas (Gramaje *et al.* 2011; Urbez-Torres 2014). There are also some suggestions that the fungi associated with Petri disease/Esca complex are mostly endophytes and the symptom expression seen in the field is triggered by biotic and abiotic factors such as water stress and extreme temperatures (Fischer *et al.* 2019; Hrycan 2020). Thus, it is possible that these YVD-associated microorganisms are merely part of the whole plant microbiome, however, stress factors may influence their transition from an endophytic to a pathogenic phase. Since there is increasing concern about GTD spread in nurseries, future studies to establish the infection thresholds of these YVD-associated fungi in propagation materials that lead to subsequent disease in the vineyard is vital.

11.1.4.2 Hot water treatment

This study further investigated the effects of HWT on the abundance and diversity of microbial populations in young vines and focused on dormant one-year-old grafted and own-rooted vines from Nursery 2 that participated in the first survey. The short duration HWT of 54°C for 5 min used in this study was based on the protocol aimed at eliminating external contamination in nursery vines (Standards Australia AS 5588-2013, 2013). Understanding the effects of stress, potentially attributed to HWT, on the overall total health status of young vines will provide some insights on the importance of this practice in the quality management of plant materials in nurseries. To our knowledge, this study is the first attempt to investigate the effects of HWT on the diversity of endophytic microorganisms of nursery plants in Australia.

Young dormant vines harboured abundant microorganisms regardless of plant type or variety. Similar to the first nursery microbiome study, the fungal communities were dominated by environmental fungi of unknown importance to viticulture, while a small proportion were identified as YVD pathogens, beneficial fungi and bunch rot pathogens. Bacterial communities were abundant across plant types and varieties, with the Pseudomonadaceae and Bacillaceae being the two most abundant bacterial families.

The short duration HWT treatment did not cause obvious alteration in the fungal community composition of young vines, regardless of plant type or varieties. The YVD pathogens were equally abundant in both HWT and NHWT vines, suggesting that the short-duration HWT was ineffective in reducing these fungal pathogens. In contrast, HWT was found to alter bacterial compositions in young vines by favouring the heat-tolerant Bacillaceae, while supressing Pseudomonadaceae populations in certain varieties. The increase in Bacillaceae was particularly evident in Paulsen roots after HWT although a slight increase was also observed in Shiraz and Sauvignon Blanc. The suppression of Pseudomonadaceae populations by HWT was more evident in Shiraz vines, suggesting this variety might be more sensitive to HWT. Since *Bacillus* species are soil-inhabiting bacteria that produce heat-resistant spores, their increase in abundance in HWT vines suggests that high temperatures can promote spore germination in this bacterial group. Some Bacillus strains isolated from the inner tissues of grapevine were found to promote plant growth (Baldan *et al.* 2015) and biocontrol agents against plant pathogens (Kamal *et al.* 2016). Pseudomonas bacteria are also known for their biological control activity. One strain of *P. poae* isolated from grapevine wood was found to be antagonistic to GTD pathogens *in vitro* and *in planta* (Niem *et al.* 2020a, 2020b). It is also important to note that the Bacillaceae were in very low abundance in materials from Nursery 2 in the previous microbiome studies where the vines for this HWT experiment were procured. This suggests that their relative abundance may be influenced by other factors such as climate and season. Future research to investigate the functionality of these bacterial strains will provide insights on the benefits on these beneficial microorganisms on the health status of the vines.

The lack of obvious effects of HWT on the fungal communities in this study is similar to the results from studies conducted overseas. A study in the Czech Republic and Spain also revealed that YVD pathogens were able to tolerate more stringent HWT of 50-53°C for 30 min (Eichmeier *et al.* 2018). A separate study in Spain also showed that HWT at 53°C for 30 min only reduced the abundance, but did not eradicate *C. luteo-olivacea*, *P. chlamydospora* and *N. parvum* (Lade *et al.* 2022b). Both studies conclude that these stringent HWT protocols were not completely effective in eliminating these pathogens. However, the above studies only investigated the impact of HWT on fungal communities in young vines, thus, information on the impact of the longer duration HWT on bacterial communities needs further investigation.

Standards for grapevine propagation materials in Australia and New Zealand have been established, although testing for GTD pathogens is not included in these standards to date. The Australian Standard for Grapevine Propagation Material (Standards Australia AS 5588-2013, 2013) recommends the use of HWT at 50°C for 30 min as an internal treatment to reduce crown gall and trunk disease pathogens. The standard also recommends the short duration HWT of 55°C for 5 min for the external treatment of surface fungi, bacteria, phylloxera and nematodes. However, HWT is not highly recommended in New Zealand due to issues with poor growth and vine deaths that were associated with this practice (New Zealand Winegrowers Grafted Grapevine Standard V4.0, 2021). Since recent microbiome studies raised questions on the efficacy of the most common HWT protocol 50°C for 30 min used in commercial nurseries (Fourie and Halleen 2004b; Halleen *et al.* 2007; Gramaje *et al.* 2009, Gramaje and Armengol 2011; Bleach *et al.* 2013), the practice of HWT to reduce YVD pathogens in nurseries should be used with caution, due to its uncertain effects on the overall microbial population of the vines that may influence its overall health status, as well its limited efficacy against grapevine trunk disease pathogens.

In summary, the two microbiome studies provided some initial insights on the microbial communities that are naturally inhabiting young vines from different Australian nurseries. A diverse population of the microbial communities was present in grapevine materials from different nurseries. These studies further revealed that one of the current HWT treatment protocols used for dormant vines in Australia was ineffective in altering fungal populations but caused some variation in abundance for certain bacterial groups. Thus, investigation on the functionality of these bacterial communities is necessary to understand their role in vine health. The results generated from these studies are crucial first steps in understanding the current health status of grapevine planting materials in Australian nurseries. The new knowledge generated from this study may lead to developing new nursery practices that nurtures the abundance of key beneficial microorganisms associated with young vines.

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11.2 Infection thresholds of Botryosphaeria dieback in nursery propagation material

11.2.1 Introduction

Botryosphaeria dieback (BD) is a serious disease of grapevines worldwide, causing cankers, dieback and death of vines. Along with Eutypa dieback (ED), BD was ranked in the top five priority diseases of the Australian wine grape industry (GWR 08/04; Scholefield *et al.* 2010). It was previously thought to be a problem in older and mature vines as they were initially found to be associated with vines older than eight years of age (Larignon and Dubos 2001, Larignon *et al*. 2001). An investigation into the prevalence and distribution of Botryosphaeriaceae species in grapevine propagation nurseries in New Zealand revealed young, grafted plants, and cuttings from eight out of nine nurseries were positive to BD pathogens (Billones-Baaijens *et al*. 2013a). These pathogens were mostly detected in asymptomatic samples of rootstock and scion canes, and grafted plants.

The study in New Zealand suggests some nursery plant material with latent infections of BD can be unwittingly sold to vineyards, sometimes leading to dieback or even death of young vines (Billones-Baaijens *et al*. 2013b). However, the link between these latent infections and BD symptoms observed in vineyards is still unclear. Studies have also shown that water stress can increase the susceptibility of young vines to grapevine trunk diseases (GTDs). According to Slippers and Wingfield (2007), disease expression for Botryosphaeriaceae species has been linked to stress factors such as drought or water deficit. Amponsah *et al*. (2014) showed dieback symptoms developed in mid-winter when vines were given too much water (100% moisture) or too little (15% moisture) making them more susceptible to infection. An increase in susceptibility to some species of Botryosphaeriaceae was observed in glasshouse potted vines under restricted water regime (Qiu *et al*. 2016). In contrast to the above-mentioned studies, water deficit did not increase the susceptibility of vines to *D. seriata* in a low-rainfall region in the Riverland, SA (Sosnowski *et al*. 2021).

Information on the prevalence and distribution of Botryosphaeriaceae species in Australian nurseries is, currently, limited. A survey focused on Petri disease found Botryosphaeriaceae to be present in young vines and propagation materials from Australian nurseries but this study did not fully investigate the impact of these fungal species on the health of young grapevines (Edwards and Pascoe 2004). Sampling from the Riverina region in New South Wales (NSW) also showed 10% of the young vines from one nursery and 25% of the samples from one mother vine block tested positive to the BD pathogen, *Diplodia seriata* (Wine Australia, 2013). This species and the black foot pathogen, *Ilyonectria macrodidyma*, were also associated with one month to eight-year-old vines showing decline, based on a survey of 20 young vineyards in NSW (Whitelaw-Weckert *et al*. 2013), suggesting some of these Botryosphaeriaceae species may have originated from nurseries.

The role of grapevine propagation in the spread of BD requires further investigation, particularly with regard to understanding the threshold of infection and abiotic factors that lead to vine decline. However, accurate quantification of infection severity from naturally infected young vines is difficult, since latent infections have no external symptoms and are randomly distributed within a vine (Billones- Baaijens *et al*. 2015). In June 2017, a preliminary experiment was undertaken to produce young vines naturally infected with BD from cuttings collected from a commercial vineyard. Due to low levels of natural infection, to proceed with pot experiments, a published inoculation method to standardise the severity of BD infections in vines that can be accurately quantified using molecular methods was established. This method is essential for the production of vines with prescribed levels of infection required for the investigation of the effects of abiotic stress on the disease expression of BD in young vines.

This preliminary study aimed to investigate the infection thresholds of BD infections in propagation materials and the effects of water stress in the disease expression of young vines. An inoculation method using cuttings was developed, to simulate natural infections and standardised BD infection in young vines. This method allowed the production of experimental vines with different levels of infection that were accurately detected and quantified using molecular tools. The study further involved the quantification of BD pathogens in young vines, followed by planting under water-stress conditions. This study may provide insight on the infection thresholds for BD pathogens required for disease expression in vines.

11.2.2 Methods

11.2.2.1 Evaluation of inoculation method to standardise levels of infection

Vacuum inoculation of dormant canes using fluorescent pigment

Dormant Shiraz canes were collected in June 2017 from a commercial vineyard at Hilltops, NSW and assessed for BD infections by fungal isolations and qPCR. Of the 400 cuttings analysed, only 1% tested positive to BD infections, thus providing insufficient levels of infection for the pot experiments. To simulate natural infection, the vacuum inoculation method developed by Rooney and Gubler (2001) was evaluated for its suitability to inoculate BD pathogens into the grapevine canes.

The method was initially evaluated using a 10% solution of a yellow fluorescent pigment (189 Fluro Liquid Suspension Concentrate, Topline Paint Pty Ltd). Five dormant canes (cv. Shiraz; ~50 cm) were trimmed by cutting ~ 0.5 cm of tissues from the basal and apical end to create fresh wounds. Vacuum inoculation was performed using a lab bench vacuum pump (100 L/min; D.V.P. Vacuum Technologies, Taiwan) by dipping the base of the cane into the 10% pigment solution and the vacuum tube was inserted onto the apical tip of the cane and vacuumed for 10 seconds (Figure 11.8, Rooney and Gubler 2001).

To assess the translocation of the inoculated pigment, canes were sequentially cut in cross-section (~2 cm) from the base to the tip. Each section was visually assessed for yellow fluorescence using a stereo microscope with a UV light attachment. To assess the distribution of fluorescence within the cane, four to five sections from each cane were further cut into longitudinal sections.



Figure 11.8. Vacuum inoculation method to standardise the inoculum levels of dormant canes.

Preparation of spore suspensions

The BD pathogen, *Neofusicoccum luteum*, which is known to produce a yellow pigment in agar was used for inoculation. Two isolates of *N. luteum* (DAR81013 and DAR 80983; Wunderlich *et al*. 2011) were grown in prune extract agar (PEA) and incubated at 25°C with 12 h photoperiod for 10-14 days until fruiting bodies developed. To harvest the spores, ~5 mL of sterile ¼ strength Ringer's solution (Sigma Aldrich, USA) was added onto the PEA plate. The fruiting bodies were scraped from the surface of the agar and transferred into a sterile mortar and pestle and ground to release the spores. The spore suspension was passed through two layers of sterile Miracloth (Merk Millipore) to filter out the mycelial fragments and agar. Spore suspensions from each of the two isolates were combined and three concentrations (1×10^5, 1×10^4 and 1×10^3/mL) of the mixed suspension (~200 mL each) were prepared using a haemocytometer. For each concentration, 10 mL suspensions were aliquoted into 50 mL containers (10 each) and used for vacuum inoculations.

Vacuum inoculation of canes using spore suspensions

Preliminary tests using the 10% fluorescent pigment indicated the vacuum inoculation method was suitable for inoculating liquid suspensions in dormant canes and was therefore used to inoculate *N.*

luteum spore suspensions. Apparently healthy dormant Shiraz canes containing five nodes of similar length (~50 cm) and diameter (~1 cm) were collected from the same commercial vineyard at Hilltops, NSW in June 2018 and stored at 4°C for two weeks until processed. Canes were vacuum-inoculated with three concentrations of *N. luteum* spores (1×10^5, 1×10^4 and 1×10^3/mL) in July 2018 following the methods developed by Rooney and Gubler (2001), as described for the fluorescent pigment. Prior to inoculation, canes were dipped in 0.5% sodium hypochlorite for 2 min and rinsed twice with tap water before trimming the apical and basal ends (~5 mm) to create fresh wounds. Inoculations were undertaken in batches of 10 canes by dipping the base of each cane into the 10 mL spore suspensions for each concentration. The amount of suspension drawn in by 10 canes was calculated by deducting the remaining suspension from the original volume of 10 mL. The average volume of spore suspension used for inoculations was 300 µL/cane, which was equivalent to 300, 3000 and 30,000 spores/cane for low, moderate and high inoculum, respectively. The sterile ¼ strength Ringer's solution was used to inoculate negative control canes. Approximately 100 canes were used for each inoculation treatment. Four canes per inoculation treatment were randomly sampled and processed for fungal isolations and qPCR analysis while the remaining canes were rooted and used for pot experiments.

11.2.2.2 Validation of the vacuum inoculation method

Detection of the pathogen by fungal isolations

To validate whether the vacuum inoculation method was successful in infecting the canes, four canes per treatment were randomly selected and placed in individual plastic bags sprayed with sterile deionised water (SDW) to provide humidity, incubated at 25°C and 12 h photoperiod for two weeks and used for fungal isolations and DNA extractions. The 14-day incubation was essential to provide ideal conditions for the development of infection.

To investigate the distribution of infection within the cane, each cane was trimmed (~5 mm) at the base and tip before single-node sections (~10 cm) were excised from the base, middle and apical end of each cane and labelled accordingly (Figure 11.9a). Each section was peeled to remove the bark and surface-sterilised using 70% EtOH for 2 min, and rinsed twice with SDW for 2 min. After surface-sterilisation, each section was further cut into smaller fragments (Figure 11.9b) using a sterile coffee grinder. Ten pieces of the wood chips (~5 mm) were randomly picked and plated onto two PDA-C plates with five wood chips per plate (Figure 11.9c). Plates were incubated at 25°C in total darkness for 3-7 days and observed for the growth of *N. luteum*. Disease severity was calculated as the percent of the number of wood chips positive to *N. luteum*. The remaining wood chips were placed into 2 mL sterile tubes and stored at -80°C until processing for DNA extractions (Figure 11.9d).

DNA extractions from inoculated wood

Approximately 1 g of wood chips were placed in a sterile 2 mL tube and stored at -80°C until required for DNA extractions (Figure 11.9d). All frozen samples were freeze-dried (Christ, Germany) for 36 h before homogenising using 10 mL grinding jars attached to a TissueLyser II (Qiagen, U.S.A.) at 20 Hz for 2 min 30 s. The pulverised wood (100 mg) was transferred into a sterile 2 mL tube and used for DNA extractions.

DNA was extracted from the pulverised wood samples using the Qiagen Plant Pro DNA extraction kit (Qiagen, U.S.A.) using the manufacturer's protocol. All DNA samples were eluted to a final volume of 100 µL using DNA-free water and were quantified using a Quantus™ Fluorometer (Promega, U.S.A.) prior to qPCR.

The inoculated canes were further assessed for *N. luteum* infection at post-rooting by qPCR. The four sub-sampled vines per treatment were collected, washed and stored at 4°C for 3-4 days until processed. The samples were processed as previously described for the pre-rooting samples and used for DNA extractions and qPCR.

To determine if the pathogen was translocated into the roots and shoots, four separate rootlings per treatment were further assessed. The roots from each plant were thoroughly washed with tap water to remove soil before cutting into ~2 cm length and used for DNA extractions. The top two shoots for each rootling were also collected and cut into ~2 cm lengths and used for DNA extractions. All tissue samples were processed for DNA extractions using the same methods as the cane samples, except that 50 mg of freeze-dried sample was used instead of 100 mg.

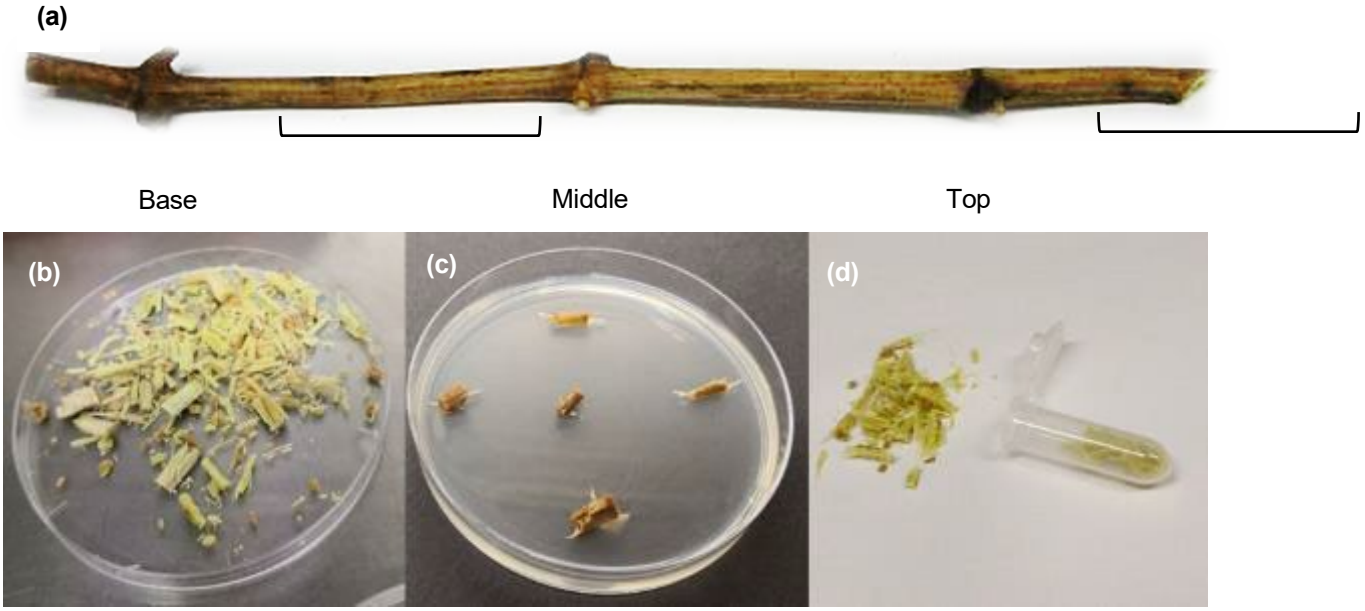


Figure 11.9. Methods to assess *Neofusioccum luteum* infections from the vacuum-inoculated canes: a) representative cane indicating the position of the tissues used for the assessment; b) wood samples homogenised into smaller chips prior to fungal isolations and DNA extractions; c) randomly selected wood chips plated onto PDA-C plates at five pieces per plate; and d) wood chips used for DNA extractions and quantitative PCR analysis.

Quantification of *Neofusicoccum luteum* DNA from wood samples by duplex quantitative PCR

The sub-samples from vacuum-inoculated wood were analysed using duplex qPCR. The Botryosphaeriaceae multi-species primers and hydrolysis probe developed by Billones-Baaijens *et al*. (2018) were used as the primary primers to quantify *N. luteum* DNA from the inoculated wood. A second set of primers and probe (AcvitIF - Acvit1R and AcvitTaq probe), which was developed (R. Baaijens 2019, unpublished) to amplify a 106 bp fragment of the *V. vinifera* actin gene, was added to the reaction as an internal control. The Taq-Bot probe was labelled at the 5' end with 6-carboxyfluorescein (FAM) while the AcvitTaq probe was labelled at the 5' end with Hexachlorofluorescein (HEX). Both probes had a double-quencher (internal Zen with 3' Iowa Black® FQ; Integrated DNA technologies, USA).

All qPCR assays were performed using a RotorGene 6000 (Corbett Life Science, Qiagen, U.S.A.). Each 20 µL reaction contained 10 µL of 2× GoTaq® Master Mix (Promega, U.S.A.); 500 nm of each primer, 250 nm of each probe and 5 µL of template DNA at four technical replicates per sample. All qPCR assays 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. The TaqBot probe was detected using the green channel at 510 nm while the Actvit Taq probe was detected using the yellow channel at 555 nm. For each assay, three controls were included: 1) non-template control (H_2O); b) standard (500 pg) Bot-Btub gBlock (Billones-Baaijens *et al*. 2018); c). standard (500 pg) Acvit gBlock.

The standard curves developed by Billones-Baaijens *et al.* (2018) were used to determine the amount of *N. luteum* DNA that was amplified by each qPCR assay. The standard curve was imported into the Rotor-Gene Q software (Version 2.3.1) and calibrated using the standard (Bot-Btub gBlock, 500 pg) that was included in each qPCR assay. The resulting regression equations were used to quantify the number of *N. luteum* β-tubulin gene copies in each reaction as previously described by Billones-Baaijens *et al*. (2018) following the MIQE guidelines (Bustin *et al*. 2009).

Since DNA yield varied between wood samples (100 mg) used for extractions, the quantification of pathogen DNA per sample was normalised by calculating the total number of gene copies per ng of DNA using the following formula below:

$$N = Q/(D*T)$$

Where:

N	=	Calculated no. of gene copies per sample
Q	=	The mean number of gene copies detected by qPCR
D	=	Total DNA yield (ng) from 100 mg of wood
T	=	DNA volume (5 µL/reaction) used as template in one reaction

11.2.2.3 Infection threshold experiments using vacuum-inoculated vines

The effect of water stress on the symptom development of young vines inoculated with different BD inoculum levels was investigated. This work aimed to provide insight on the infection thresholds that may result in the expression of BD disease in young vines.

Preparation of potted vines for experiments

The dormant canes previously vacuum-inoculated with 300, 3,000 and 30,000 *N. luteum* conidia (~90 canes/treatment) were rooted in biodegradable Jiffy pots containing moist seed and cutting raising mix (Osmocote, Australia). The Jiffy pots were placed in plastic tubs at 24 pots per tub with ¾ of the basal end of pots covered with moist perlite to maintain constant temperature throughout the root zone. The tubs were placed on top of heat mats (27-35°C) and allowed to root inside a dark shed at ambient winter temperature (5-17°C) for five weeks (Figure 11.10). After five weeks, when buds and roots started to develop, all tubs were transferred to the glasshouse (17-27°C) and kept for another two weeks to allow the vines to acclimatise. All canes were assessed for root and shoot development and the percent strike rate was calculated. Vines with at least two shoots and well-developed roots were selected before transplanting into 20 and 30 L plastic pots and used to establish greenhouse and shade house experiments, respectively. A commercial potting mix (60% compost, 20% washed sand, 20% screened loam) was used to grow all vines. From the remaining vines, four vines per treatment were randomly selected and further used for qPCR analysis to detect the presence of *N. luteum*.



Figure 11.10. Inoculated dormant cuttings rooted in commercial seed raising mix and placed on top of heat pads (indicated by arrows) to induce rooting.

Greenhouse pot experiment

For the greenhouse experiment, vines from different inoculation treatments were randomly assigned to two watering regimes (full and water deficit) at 15 vines per inoculation × water regime treatment combination. Potted vines were placed on metal benches and arranged in randomised complete block

design (RCBD) at five replicate vines per treatment in each block (Figure 11.11a). The full water regime was designed to water the pots to the point of first visual free water draining after each irrigation, while for the water-deficit regime, only 30% of the water was provided. Pressure compensated drip emitters were used for the full (8 L/h) and water deficit (2 L/h) regimes. One day prior to applying the water regimes, all vines were given full water followed by irrigation at three times a day (0700, 1400 and 1900 h) for 3 min throughout the growing season (September-April). The drip emitters were recorded to deliver approximately 1200 mL/day and 360 mL/day for full and water deficit regimes, respectively.

All vines were maintained at ambient temperature of 17-27°C during the growing season. The top two shoots were selected and vertically-trained using bamboo stakes while any remaining shoots were removed. Shoots were maintained to ~1.5 m height and any lateral shoots were removed during the duration of the experiment. Slow-release fertiliser (~75 gm/pot, Osmocote, Scotts Australia Pty, Ltd) was applied to each vine every six months. A sulfur vaporiser was installed inside the greenhouse, programmed to emit sulfur every 12 h to prevent powdery mildew and mite infestations.

In winter, the heating and irrigation systems were switched off and air vents were opened to reduce the internal ambient temperature to 5-17°C, allowing the vines to go dormant. Dormant vines were pruned to two nodes per cane that carried four buds the following season. In spring, the new season shoots were further trained and maintained as described in the first season.

Shadehouse pot experiment

For the shadehouse experiment, vines from different inoculation treatments were also randomly assigned to two watering regimes at 14 vines per inoculation × water regime treatment combination. All vines were placed on the concrete floor and arranged in a RCBD with two replicate vines per treatment in a row (Figure 11.11b) for a total of seven rows. Water regimes were similar to the greenhouse experiments except the drip emitters used were at 10 L/h and 2 L/h for the full and water deficit treatments, respectively. All vines were also manually provided with full water one day prior to water treatments followed by irrigation at three times a day (0700, 1500 and 2300 h) for 3 min in spring (September-November) and autumn (March-May) and four times a day (0700, 1300, 1900, 0100) for 5 min in summer (December to February). The drip emitters were recorded to deliver approximately 1500 mL/day (spring and autumn) and 3300 mL/day (summer) for the full water regime and 360 mL/day (spring and autumn) and 480 mL/day (summer) for the water deficit regimes. All vines were maintained outdoors, and shoots were vertically trained on metal wires and trimmed to ~1.5 m height, similar to the greenhouse vines. Slow-release fertiliser was applied to each vine every six months as described for the greenhouse experiment. Vines were sprayed with wettable sulfur (18 g/L) every 3-5 weeks to prevent powdery mildew and mite infestations. In winter 2019, vines were pruned to three nodes per cane that carried six buds the following season. In spring, the new shoots were further trained and maintained as described in the first season.



Figure 11.11. Pot experiments in the greenhouse (a) and shadehouse (b) using vacuum-inoculated vines to investigate the effects of water deficits on Botryosphaeria dieback symptom expression.

Water stress assessment

The soil moisture content for each pot was monitored weekly using a ThetaProbe attached to a HH2 hand-held moisture reader (Delta-T Devices, Cambridge, England) during the growing season (September-May) for all glasshouse and greenhouse vines. Theta probes were inserted into the potting mix and moisture was measured at three different points and the average soil moisture was calculated for each pot. Due to disruptions from the COVID-19 pandemic and subsequent lockdowns, the soil moisture monitoring was only performed until March 2020.

In mid-December 2018 to late January 2019, extreme high temperatures of 40°C or higher were recorded for 14 days. To minimise heat stress and sunburn on shadehouse vines, extra shade cloths were installed in early January and removed in March 2019. These extra shade cloths were again installed in the following summer to minimise heat stress. In January 2020, the irrigation malfunctioned for three days when maximum temperatures ranged from 30-38°C, causing the vines to defoliate. All vines were provided with full water regime until they fully recovered approximately 14 days later, following which full and deficit water regimes were reinstated.

To confirm the efficacy of the water treatments, the basal end of the topmost pruned canes for each season were collected during pruning in winter 2019 and 2020 and processed for carbon isotope composition ($\delta^{13}C$) analysis. The carbon isotope composition ($\delta^{13}C$) of grapevine tissues can be used to give an integrated measure of water stress, either during the ripening period if berry sugars are analysed or spanning a longer period of growth for vegetative parts of the vine (Van Leeuwen *et al.* 2009, Taskos *et al.* 2020). For each treatment, four samples from different vines were pooled inside a paper bag for a total of three pooled samples per treatment. All samples were placed in a drying oven at 65°C for one week. All pooled samples were homogenised into a powder and stored at 30°C. All samples were sent for analysis at the Stable Isotope Laboratory, Research School of Biology, The Australian National University (ACT) in December 2020 to determine the carbon isotope composition ($\delta^{13}C$) of the wood dry matter following the methods described by (Farquhar and Richards, 1984, Gibberd *et al.* 2001).

In summer 2020 and 2021, all berries from each vine were harvested and bunch number, average yield and berry weight for each treatment were recorded and data between treatments were compared.

Botryosphaeria dieback symptom assessment

All vines were visually monitored fortnightly for BD symptoms throughout the growing season. In winter 2020, wood samples from the original inoculated canes were collected by cutting the top end of the trunk ~1 cm above the second node. All pruning wounds were treated with A.T.C.S. pruning sealant using a paint brush. For greenhouse vines, (Figure 11.11a) the lower part of the trunk containing two shoots from season 2 were retained and pruned to two buds to carry four buds for the third season. For the outdoor vines, (Figure 11.11b) the lower part of the trunk containing two shoots from season 2 were further retained and pruned to three nodes to carry six buds for the third season. Sub-samples for each treatment were analysed by fungal isolations and qPCR to detect *N. luteum* using the methods described in the pre-rooting assessment.

In June-July 2021, batches of 40 vines representing five replicates per treatment were lifted out of their pots each week for a period of five weeks. All vines were washed thoroughly with tap water to remove the potting mix and other debris and stored at 4°C for 5-7 days until processed. Each vine was cut to separate the 1, 2, 3 and 4 year-old wood (Figure 11.12). The bark was removed from each tissue type (~2 cm) before surface-sterilising with 70% ethanol for 2 min, rinsed twice with sterile deionised water (SDW) for 1 min and air-dried inside the laminar flow for ~30 minutes. After surface-sterilising, the basal and distal end of the four-year-old wood (the original inoculated cane) were sub-sampled by grating each end separately using a sterile cheese grater to recover ~ 2 g of grated wood. Fungal isolations were performed for each sample by plating ~1 mg of grated tissue (4× per plate) onto a PDA-C. All plates were incubated at 25°C total darkness for 5-7 days and assessed for characteristic growth of *N. luteum*. The remaining grated tissue was transferred onto a sterile 2 mL tube and stored at -70°C until required for DNA extractions. DNA were extracted from the sub-samples for the basal end of the four-year-old wood and analysed by qPCR to detect *N. luteum* using the methods described in the pre-rooting assessment.

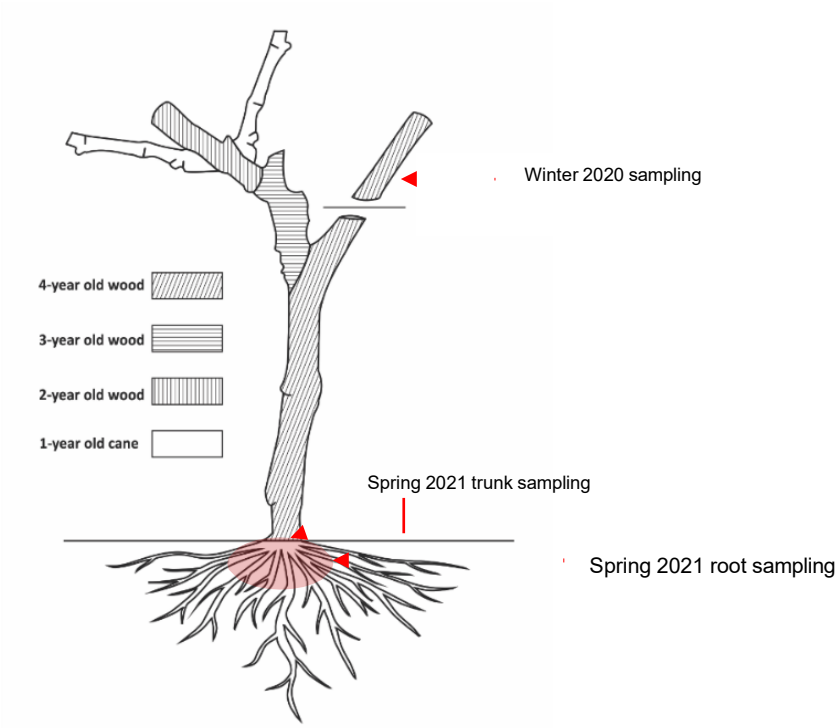


Figure 11.12. Schematic diagram of the vacuum-inoculated vine showing the positions of the tissues sampled. The tissue samples used for the assessment by fungal isolations and/or quantitative PCR are indicated by arrows. (Illustration by Edgar Billones)

To further determine whether the pathogen was translocated into the roots and shoots, sub-samples from basal end of the 1, 2 and 3-year-old wood that spanned the interface between each tissue age were also collected by grating ~1 g of wood as described above. All grated samples were stored in 2 mL tubes at -70°C until further use. Three-year old roots (~ 5 g) closest to the base of the four-year-old trunk were further cut into 5 mm sections and surface-sterilised as above. All root samples were stored in 5 mL plastic tubes at -70°C until further analysis.

Due to the high number of samples and limited time, the qPCR assessment for all vines were only performed for the basal and apical end of the four-year old original inoculated canes. Representative root samples from 10 vines that tested positive to the pathogen DNA (>200 copies) were further analysed by qPCR following the methods described for wood samples. The remaining samples were stored at - 70°C for future analysis when time and resources are available.

Data analysis

All qPCR data were expressed as number of β-tubulin gene copies per nanogram of DNA used as template in each reaction. Due to lack of significant differences between sampling times, the qPCR data from the 2020 (distal end) and 2021 sampling (basal end) of the original inoculated cane were combined and analysed using IBM SPSS Statistics 27. Effects of water stress and pathogen inoculation for each treatment were determined by analysis of variance (ANOVA) and a pair-wise comparison between means was performed using least significant difference (LSD) at the 5% level. The effects of water stress and pathogen inoculation on yield, bunch numbers and berry weight were further determined using ANOVA as described for qPCR.

11.2.3 Results

11.2.3.1 Development of inoculation method to standardise *Neofusicoccum luteum* infections

Vacuum inoculations using fluorescent pigment

Yellow fluorescence was observed in the vascular tissues of the vacuum-inoculated canes (Figure 11.13) that showed translocation all the way from the base to the apical tip of the canes. The fluorescence was generally observed to be more intense at the basal part of the cane compared to the apical tip.

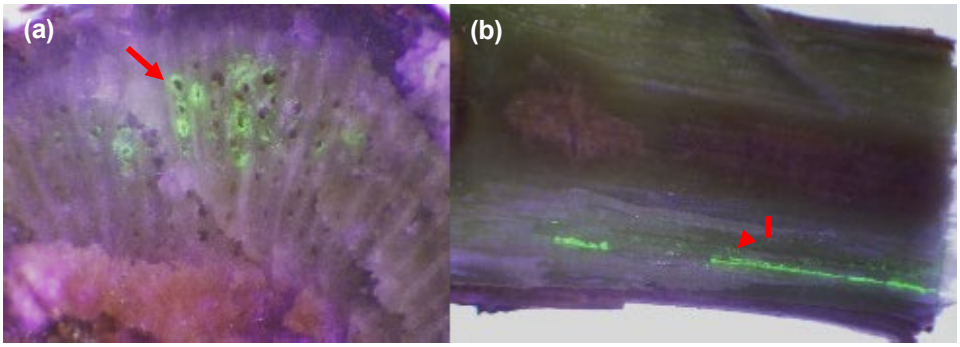


Figure 11.13. Cross-section (a) and longitudinal section (b) of grapevine cane showing yellow fluorescence in the xylem vessels (arrows) after vacuum inoculation with a fluorescent pigment.

Assessment of canes vacuum-inoculated with *Neofusicoccum luteum* spores

Assessment by pathogen re-isolation

After 14 days incubation, all sub-sampled canes inoculated with *N. luteum* conidia exhibited some brown staining in the xylem vessels regardless of spore concentrations (data not shown). No obvious wood staining was observed in any of the negative control canes (Ringer's solution only).

Re-isolations from the sub-sampled canes at pre-rooting showed that the vacuum inoculation method was successful for introducing *N. luteum* conidia into the canes, with all sub-samples testing positive to *N. luteum.* Disease severity varied between levels of inoculum and position of the tissue within the cane. Re-isolation of *N. luteum* from the wood chips differentiated the low, moderate and high inoculum, with the highest disease severity detected from canes inoculated with 30,000 conidia and the lowest from those inoculated with 300 conidia (Figures 11.14 and 11.15). For low inoculum (300 conidia), disease severity was highest on the basal end of the canes (55%) while the middle and apical end of the canes had 15 and 17.5% disease severity, respectively (Figure 11.15). For moderate inoculum (3,000 conidia), disease severity was also highest at the basal end of the cane (97.5%), followed by the top (67.5%) and middle part (45%). For the high inoculum (30,000 conidia), disease severity was similar in different parts of the cane, with highest severity at the basal end of the cane (100%), while the middle and the top had 87.5% and 97.5% severity, respectively. No *N. luteum* was re-isolated from the negative control canes (Figure 11.15).

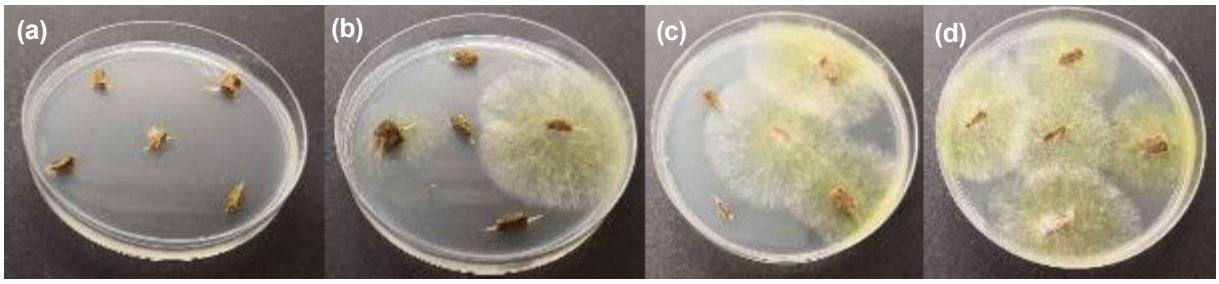


Figure 11.14. Representative photos of wood chips from vacuum-inoculated canes that were plated onto potato dextrose agar and observed for yellow pigmented mycelial growth of *Neofusicoccum luteum*. (a) negative control (Ringer's solution only); (b) low inoculum (300 conidia); (c) moderate inoculum (3,000 conidia); and (d) high inoculum (30,000 conidia).

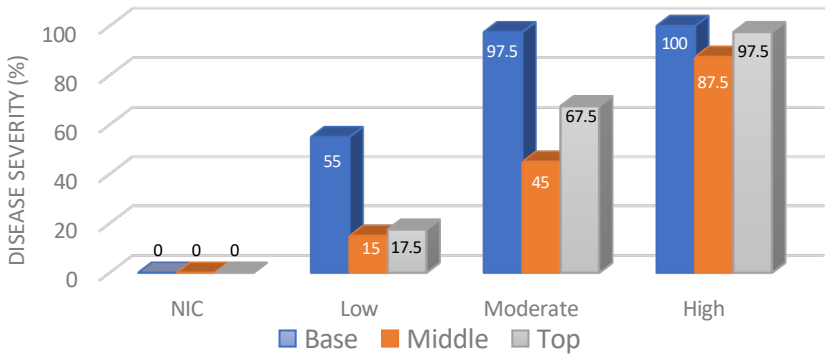


Figure 11.15. Percent disease severity of cuttings vacuum-inoculated with different concentrations of *N. luteum* spores (low – 300 conidia, moderate – 3,000 conidia, high – 30,000 conidia) based on pathogen re-isolations.

Assessment by quantitative PCR

The qPCR analyses from the sub-sampled canes at pre-rooting also showed that vacuum inoculation was a reliable method, resulting in the pathogen infecting the basal, middle and apical part of the canes when incubated for 14 days after inoculation. At pre-rooting assessment, the qPCR analyses differentiated the low, moderate and high infections, with the highest amount of *N. luteum* DNA detected from canes inoculated with 30,000 conidia and the lowest from those inoculated with 300 conidia (Figure 11.16a). Disease severity was also highest on the basal end of the canes, followed by the apical end while the middle part had the lowest disease severity. *N. luteum* DNA was not detected in any of the negative control canes.

The qPCR analyses at post-rooting, also detected *N. luteum* infecting the basal, middle and apical end of the canes, although pathogen detection was lower compared to pre-rooting, which may be attributed to the samples not being incubated for 14 days as done at pre-rooting. The qPCR analyses also differentiated low, moderate and high inoculum with the highest amount of pathogen detected from canes inoculated with 30,000 conidia and the lowest from those inoculated with 300 conidia (Figure 11.16b). *N. luteum* was not detected in the negative control canes. The roots and shoots from all treatments were also negative to *N. luteum* DNA, suggesting that the pathogen was not translocated into the roots and shoots (data not shown). It is also possible that a longer period may be required for the pathogen to translocate into the shoots and roots.

The amplification of the *V. vinifera* actin gene from all wood samples using the Acvit primers and probes was consistently observed at 15-18 cycles while no amplification was observed from any of the non-template control and Bot-gblock standards (data not shown). These results indicated that the DNA quality did not affect the efficiency and specificity of the qPCR assays.

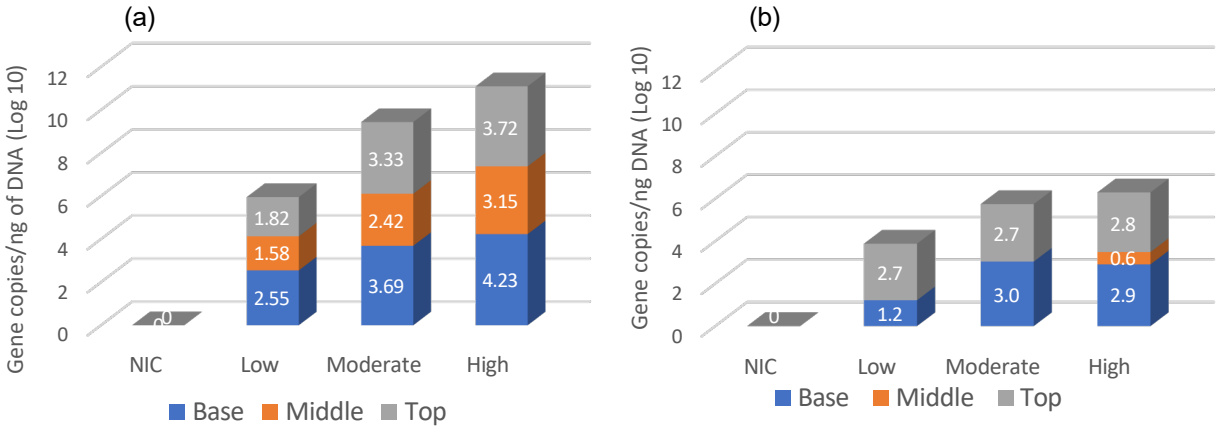


Figure 11.16. Detection and quantification of *Neofusicoccum luteum* DNA from sub-samples of vacuum-inoculated canes at (a) pre-rooting followed by incubation at 25°C for 15 days; and (b) post-rooting using quantitative PCR. The bars represent the number of copies of Botryosphaeriaceae β-tubulin gene per ng of DNA (log_{10}) collected from the apical, middle and basal ends of each cane. Canes were inoculated with either Ringer's solution only (NIC), low (300 conidia), moderate (3,000 conidia) and high inoculum (30,000 conidia).

Effect of vacuum inoculation on strike rate

The vacuum inoculation of *N. luteum* conidia on canes reduced the strike rate but there were no significant differences between inoculum levels. The strike rate for negative control canes vacuum-inoculated with ¼ strength Ringer's solution was 98% while the strike rate for canes inoculated with *N. luteum* conidia ranged from 72-77% (Figure 11.17). However, the rooting was randomised and each inoculation treatment was allocated to separate plastic tubs to prevent cross contamination. Thus, the effects of the pathogen on strike rate requires further investigation.

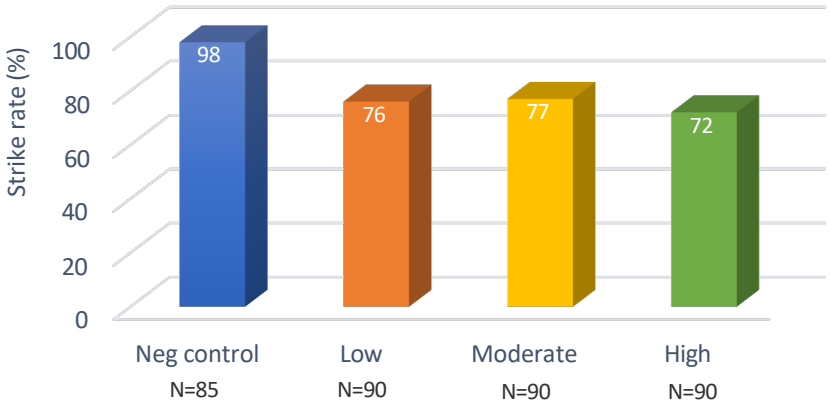


Figure 11.17. Strike rate of dormant Shiraz canes vacuum-inoculated with low (300), moderate (3,000) and high (30,000) *Neofusioccum luteum* spores. Negative control vines were vacuum-inoculated with ¼ strength Ringer's solution.

11.2.3.2 Infection threshold experiments

Botryosphaeria dieback symptom development

For the greenhouse experiments, external symptoms of BD were not observed in any of the vines during the three growing seasons, regardless of treatments.

For internal symptoms, brown staining on the central part of the wood was observed in vines inoculated with low (300), moderate (3,000) and high (30,000) *N. luteum* conidia (Figure 11.18), during pruning in July 2020. Visual assessment of the trunk cross-sections showed wood staining intensity increased with higher inoculum levels, regardless of water regime, but no obvious staining was observed in any of the non-inoculated control vines.

In winter 2021, visual assessment of the basal end of trunk cross-sections further showed wood staining intensity increased with higher inoculum levels, regardless of water regime (data not shown). The presence of *N. luteum* from stained wood was confirmed by fungal re-isolations from the tissues grated from basal end of the four-year-old wood that was originally inoculated with *N. luteum* spores. Mild vascular staining was observed in a few of the non-inoculated control vines but all were negative to *N. luteum* growth (data not shown). These results further confirmed that the vacuum inoculation method was successful in inoculating the pathogen, and grated wood can be used for fungal isolations.

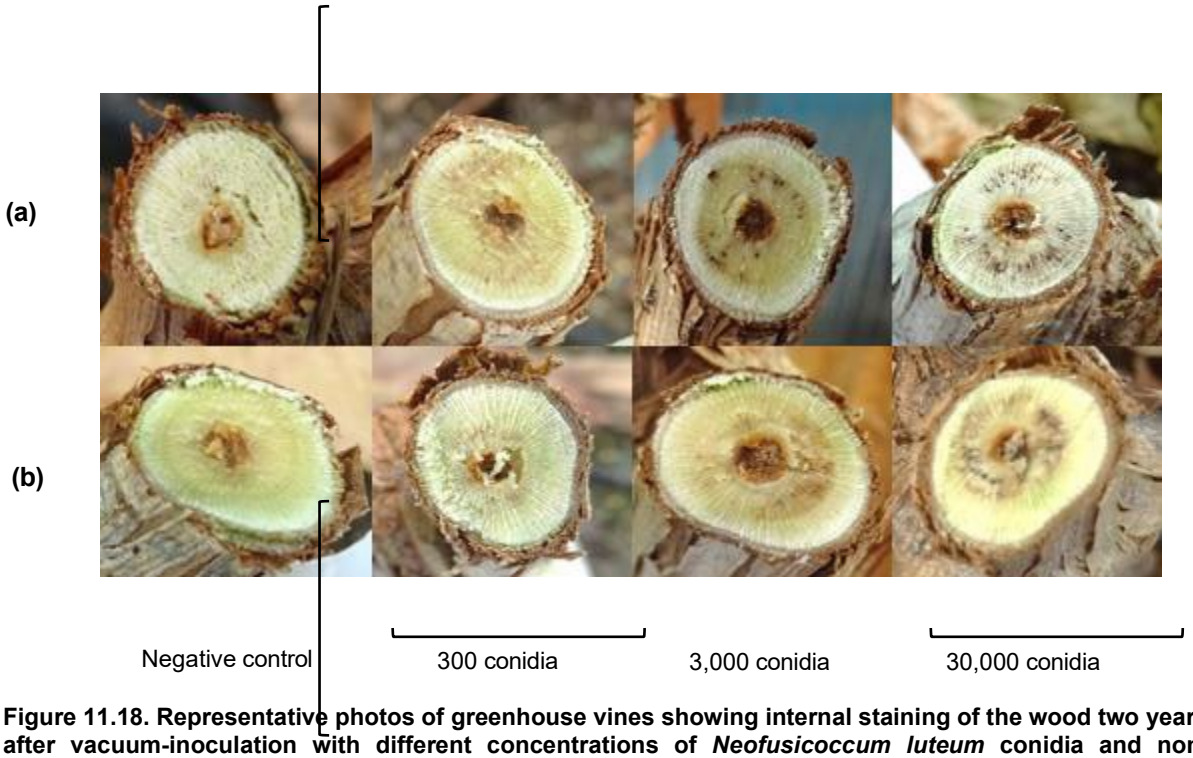


Figure 11.18. Representative photos of greenhouse vines showing internal staining of the wood two years after vacuum-inoculation with different concentrations of *Neofusicoccum luteum* conidia and non-inoculated control (Ringer's solution), and subjected to (a) water deficit or (b) full water regimes.

For shadehouse vines, no external symptoms of BD were observed in the first three growing seasons, regardless of treatments.

Brown staining in the central part of the wood was observed in vines inoculated with low (300), moderate (3,000) and high (30,000) *N. luteum* conidia (Figure 11.19) during pruning in July 2020. Visual assessment of the trunk cross-sections also showed wood staining intensity increased with higher inoculum levels, regardless of water regime. No obvious staining was observed in any of the negative control vines. The presence of *N. luteum* from stained wood was confirmed by fungal re-isolations from the sub-samples for each inoculation treatment while all negative control vines yielded no *N. luteum*.

In winter 2021, visual assessment of the basal end of trunk cross-sections showed wood staining intensity increased with higher inoculum levels, regardless of water regime (data not shown). The presence of *N. luteum* in stained wood was confirmed by fungal re-isolations from the tissues grated from basal end of the four-year-old wood that was originally inoculated with *N. luteum* spores. Mild vascular staining was observed in a few of the non-inoculated control vines, but all were negative to *N.*

luteum (data not shown). These results further confirmed that the vacuum inoculation method was successful in inoculating the pathogen into the canes (data not shown).

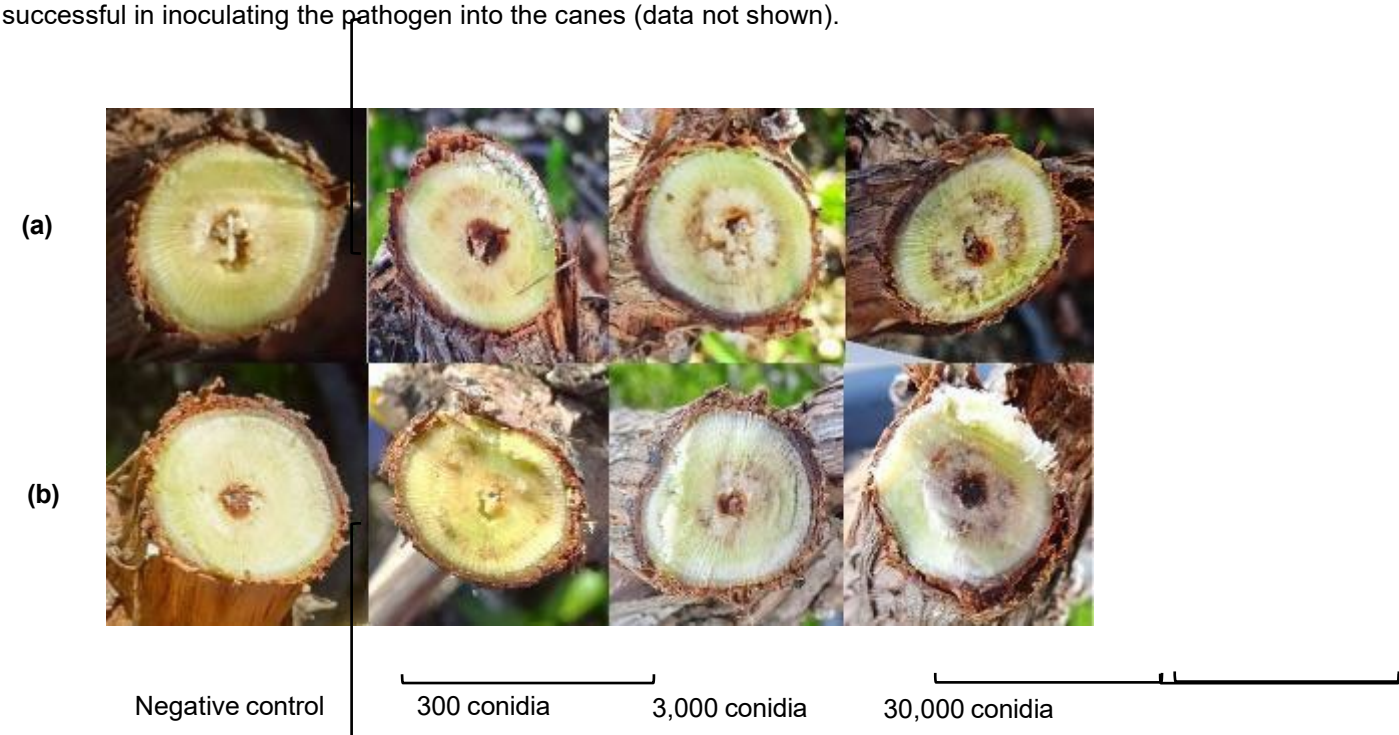


Figure 11.19. Representative photos of shadehouse vines showing internal staining of the wood two years after vacuum-inoculation with different concentrations of *Neofusicoccum luteum* conidia and non-inoculated control (Ringer's solution), and subjected to (a) water deficit or (b) full water regimes.

Assessment of Botryosphaeria infections by qPCR

For greenhouse vines, qPCR consistently detected *N. luteum* DNA from wood samples taken from the original canes that were vacuum-inoculated with different concentrations of *N. luteum* spores. No pathogen was detected in any of the non-inoculated control canes. Under greenhouse conditions, the amount of *N. luteum* DNA detected increased for vines under water deficit, but the differences were only statistically different for vines inoculated with low (300) and high (30,000) spores (Figure 11.20a). For water deficit vines inoculated with 30,000 spores, the *N. luteum* DNA was significantly higher (P=0.001) at 193 copies compared to vines under full water regime at 54 copies, which was not statistically different from the non-inoculated controls. For water deficit vines inoculated with 300 spores, pathogen DNA was also significantly higher at 97 copies compared to vines under full water regime at 33 copies, that was not statistically different from the non-inoculated controls. For vines inoculated with 3000 spores, *N. luteum* DNA was statistically similar at 144 and 99 copies for water deficit and full water regime, respectively, but were statistically higher than the non-inoculated controls.

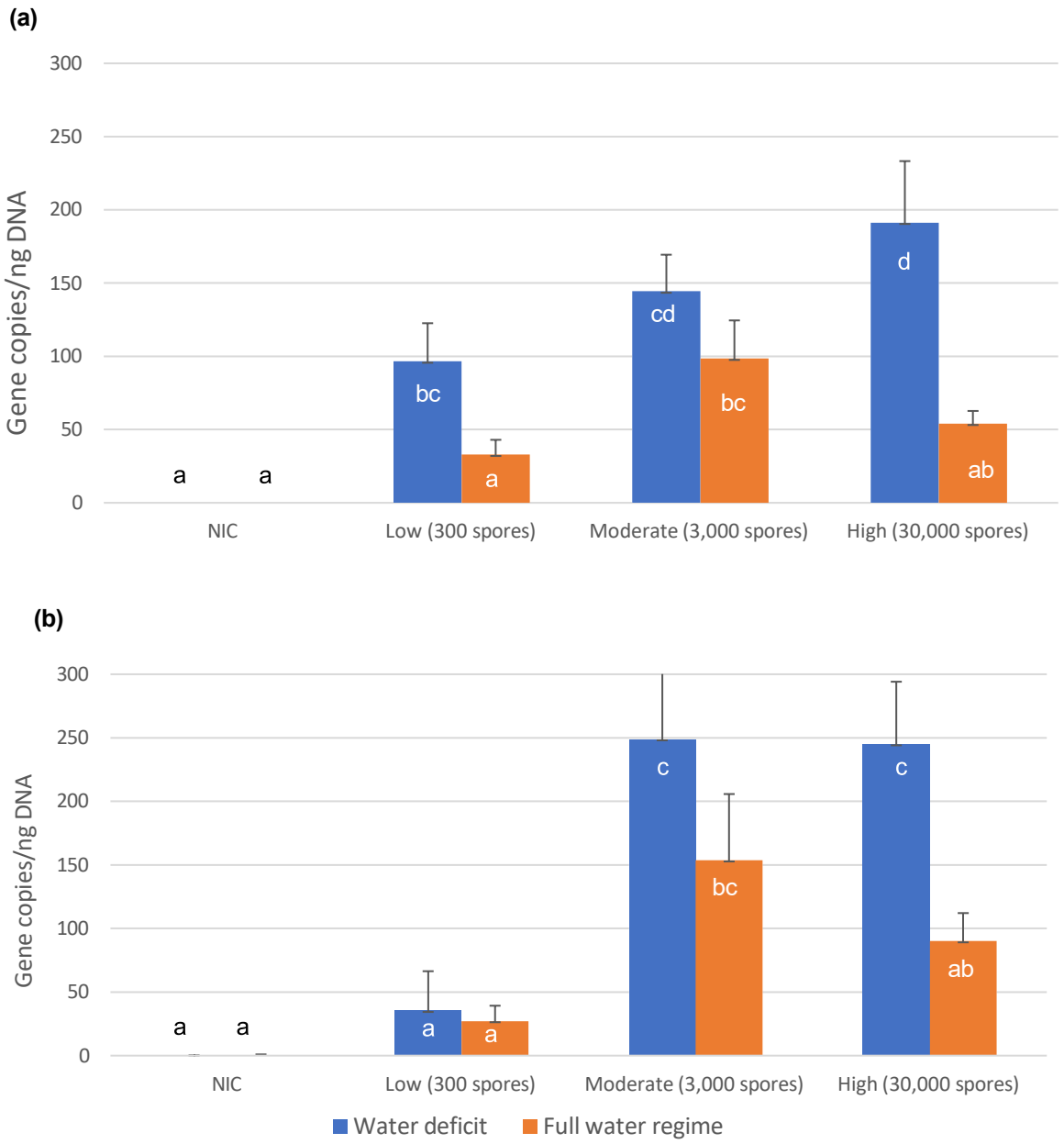


Figure 11.20. Quantification of pathogen DNA from vines in the (a) greenhouse and (b) shadehouse using quantitative PCR. Vines were vacuum inoculated with low (300), moderate (3,000) and high (30,000) *Neofusioccum luteum* spores. Non-inoculated controls (NIC) were vacuum inoculated with Ringer's solution. All vines were subjected to water deficit or full water regimes for three seasons. Bars with different letters are significantly different at *P*=0.05 LSD. Bars represent standard error of the means.

For shadehouse vines, qPCR also consistently detected *N. luteum* DNA from wood samples taken from the original canes that were vacuum inoculated with different concentrations of *N. luteum* spores. *N. luteum* was not detected in the negative control canes. Under shadehouse conditions, the amount of *N. luteum* DNA detected also increased for vines under water deficit but the differences were only statistically significant for vines inoculated with high (30,000) spores (Figure 11.20b). For water deficit vines inoculated with 30,000 spores, *N. luteum* DNA was significantly higher (*P*=0.001) at 245 copies compared to vines under full water regime at 90 copies, which was not statistically different from the non-inoculated control. For vines inoculated with 300 spores, *N. luteum* DNA was statistically similar at 35 and 27 copies for water deficit and full water regime, respectively and were not statistically different from the non-inoculated control. For vines inoculated with 3000 spores, *N. luteum* DNA was also statistically similar at 249 and 154 copies for the water deficit and full water regimes, respectively, but were statistically higher than the non-inoculated controls.

Effects of water stress on vine growth and development

The weekly soil moisture monitoring for greenhouse vines showed differences in water status between the vines subjected to full water or water deficit. For three seasons, greenhouse vines under full water regime had soil moisture ranging between 25-35% while vines under water deficit regime had soil moisture ranging 15-20% (Figure 11.21)

The weekly soil moisture monitoring for shadehouse vines also showed differences between full water and water deficit regimes (Figure 11.21). However, soil moisture varied across different periods and seasons, mainly due to variable weather conditions including rain and high temperatures in the outdoor conditions. The soil moisture for vines under full water regime ranged between 16-31% while vines under water deficit regime had a soil moisture ranging 8-20%. Extremely high temperatures during two summer seasons caused severe sunburn and defoliation in vines regardless of water regimes. In January 2020, the irrigation system also failed for three days causing all vines to defoliate. The vines recovered two weeks after they were given extra water.

For pruned canes collected during winter 2019 and 2020, Carbon isotope composition $\delta^{13}C$ values showed clear differences between vines subjected to full water or water deficit (Figure 11.22). Although there are no published values to relate $\delta^{13}C$ in canes to water status measurements, the differences were sufficient to suggest a sustained moderate water stress, based on studies where $\delta^{13}C$ of berry sugars has been compared to pre-dawn water potential. Greenhouse vines under full water regime had greater discrimination against $\delta^{13}C$ (less water stressed) compared to vines grown outside in the shadehouse, which was likely a reflection of higher stomatal conductance under more humid growing conditions.

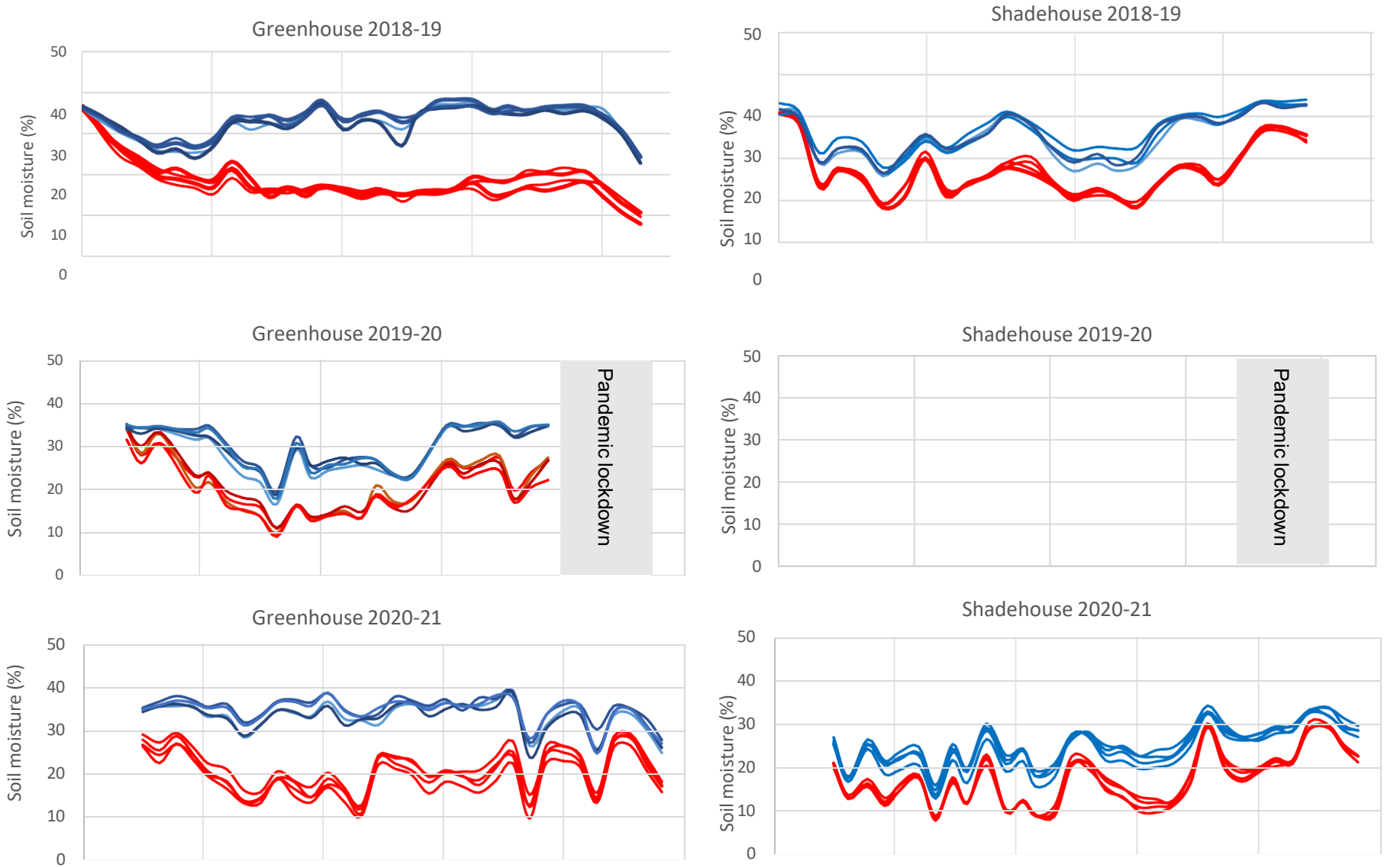


Figure 11.21. Average weekly soil moisture for potted vines in the greenhouse inoculated with low (300), moderate (3,000) and high (30,000) *Neofusicoccum luteum* spores and subjected to full (blue lines) and water deficit (red lines) regimes from November 2018 to May 2021.

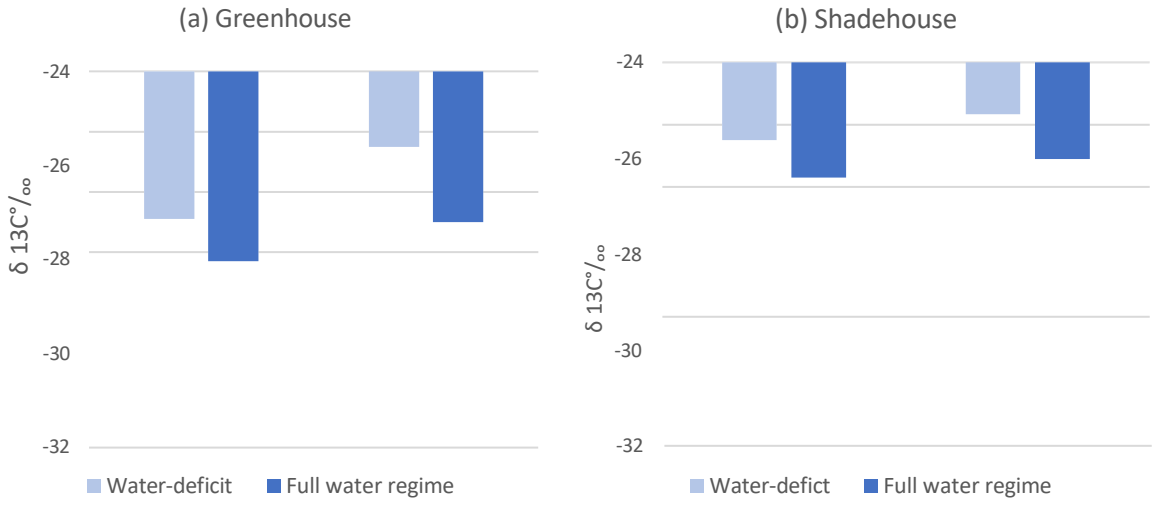


Figure 11.22. ^{13}C isotope analysis from pruned canes collected during in winter 2019 and 2020 to measure water stress from potted vines inoculated with low (300), moderate (3,000) and high (30,000) *Neofusicoccum luteum* spores and grown in the (a) greenhouse and (b) shadehouse.

Visual assessment showed that glasshouse vines under the water deficit regime generally produced smaller canes (data not shown) and roots compared to vines under full water regime (Figure 11.23). The berry production was highly variable with fewer than 20% of the vines across treatments producing berries (data not shown).

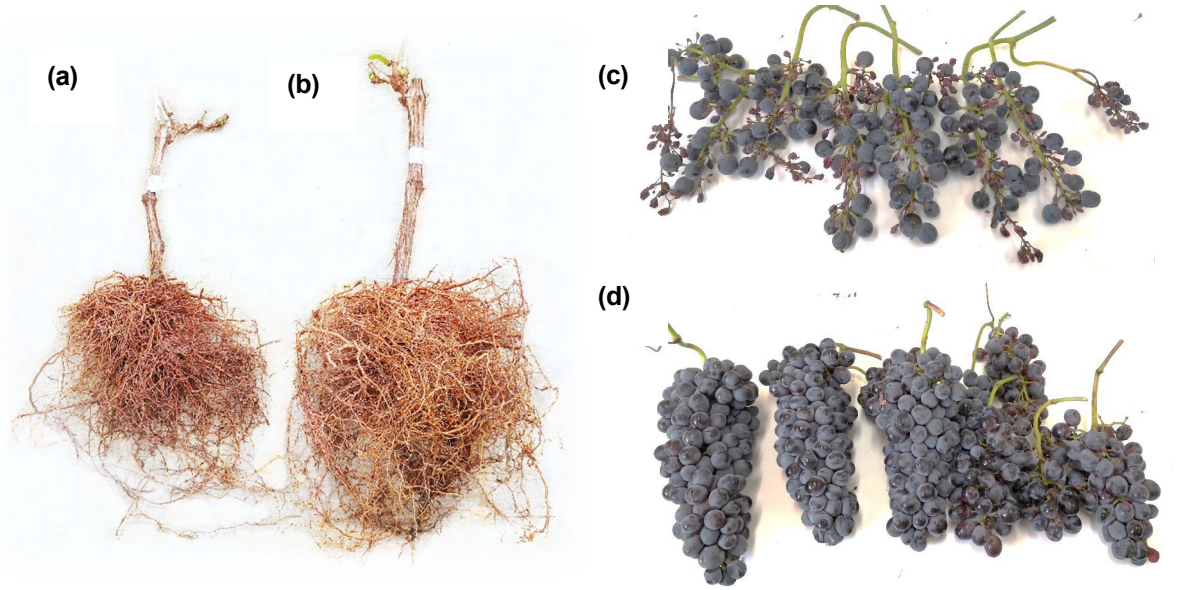


Figure 11.23. Effects of water regime on root mass and berry development. Vine under (a) water deficit regime; (b) full water regime; bunches from vines under (c) water deficit regime and (d) full water regime.

Over two years of the shadehouse experiments, the overall yield data were lower in 2020 than in 2021. (Table 11.4). For 2020, the overall yield data decreased for vines under water deficit, except for bunch number which was not significantly different (P=0.19) between vines under water deficit and full water, with an average of 4.4 and 4.2 bunches/vine, respectively. For water deficit vines, the average yield per vine was significantly lower (P<0.001) at 141 g compared to vines under the full water regime at 340 g,

regardless of inoculum level. Berries from vines under water deficit were also significantly smaller (P=0.02) with a mean weight of 1.1 g compared to vines under the full water regime, with a mean weight of 2 g regardless of inoculum level.

For 2021, the overall yield data also decreased for vines under water deficit, except for bunch number which was not significantly different (P=0.113) between vines under water-deficit and full water, with an average of 7.7 and 8.8 bunches/vine, respectively. For water deficit vines, the average yield per vine was significantly lower (P<0.001) at 1,090 g compared to vines under the full water regime at 2,000 g, regardless of inoculum level. Berries from vines under water deficit were also significantly smaller (P<0.001) with a mean weight of 1.3 g compared to vines under the full water regime with a mean weight of a 1.9 g, regardless of inoculum level.

No obvious effects on inoculum levels were observed in berry and bunch size across two seasons.

Table 11.4. Effects of water regime on the number of bunches, yield weight and berry weight for shadehouse vines.

	*No. of bunches/vine	*Mean yield/vine (g)	*Mean berry wt/vine (g)
HARVEST 2020			
Water-deficit	4.4 a	141 a	1.1 a
Full water regime	4.2 a	340 b	2.0 b
HARVEST 2021			
Water-deficit	7.7 a	1,090 a	1.3 a
Full water regime	8.8 a	2,000 b	1.9 b

*Means within a column with different letters are significantly different at P=0.05 LSD

11.2.4 Discussion

The overall aim of our research was to investigate the thresholds for BD infection in propagation materials and the effects of water stress on disease expression in young vines. This work was undertaken amid growing concerns of grapevine nursery plants being a potential source of BD infections in newly established vineyards. This emerging issue was based on previous studies conducted overseas resulting in some nursery plant materials being positive to BD pathogens (Fourie and Halleen, 2004; Aroca *et al*. 2010; Billones-Baaijens *et al*. 2013a). Billones-Baaijens *et al*. (2013b) further showed the presence of BD pathogens in New Zealand nursery plant materials, without any obvious symptoms, suggesting that these pathogens were latent. These grafted plants with latent infections can be unwittingly sold to vineyards. Thus, concern regarding the role of grapevine propagation materials in the spread of BD pathogens requires further study, particularly with regard to understanding the threshold of infection and abiotic factors that lead to symptom development and vine decline.

Many studies have shown abiotic factors, particularly water-stress, can increase the susceptibility of young vines to GTD. Disease expression for Botryosphaeriaceae species has been linked to stress factors such as drought or water deficit (Slippers and Wingfield 2007). Qiu *et al*. (2016) showed increased susceptibility in glasshouse potted vines to *D. seriata*, *N. parvum*, *B. dothidea* and *L. theobromae* under a restricted water regime. Amponsah *et al*. (2014) on the other hand, found that both too much and too little moisture can result in vines being more susceptible to *N. luteum* infection. A recent study by Galarneau *et al*. (2019) showed that *N. parvum* infections were more severe in potted vines under drought stress. In contrast to the above-mentioned studies, irrigation treatments had no significant effect on *D. seriata* wound infection, pathogen colonisation or necrotic lesions in inoculated canes under field conditions, indicating that water deficit did not increase the susceptibility to *D. seriata* (Sosnowski *et al*. 2021).

Since the abiotic factors that lead to BD symptom expression in the field remain unclear, investigations on the effects of water stress on young vines with low, moderate and high BD infections may provide insight on the infection thresholds that result in disease expression in vines. This work would involve quantification of BD pathogens from propagation materials followed by planting under water stress conditions and monitoring for disease expression. However, production of young vines naturally infected with BD pathogens was difficult since propagation materials generally have no internal or

external symptoms. These latent infections were also shown to be unevenly distributed within the vine (Billones-Baaijens *et al*. 2015), making accurate pathogen quantification difficult. Dormant canes were initially collected from a commercial vineyard to identify those with BD infections. Out of the 400 dormant canes collected, only 1% tested positive to BD infections, which was insufficient for the pot experiments. In order to generate vines for pot experiments, a vacuum inoculation method that can standardise the severity of BD infections in experimental vines and can be accurately quantified using molecular tools was evaluated. The vacuum inoculation method developed by Rooney and Gubler (2001) was initially evaluated using a fluorescent pigment for its suitability to inoculate dormant canes. Initial results showed the method was suitable for infiltrating liquid suspensions throughout the cane.

Vacuum inoculation was further tested for its suitability to infiltrate large spores such as *N. luteum* (6-12 µm; Pitt *et al*. 2020), since Rooney and Gubler (2001) developed the method for Petri disease pathogens with smaller spores (< 5 µm; Molnar *et al*. 2020). Our study showed that the vacuum inoculation method was suitable for infiltrating different concentrations of *N. luteum* spores into dormant canes without significantly impacting plant viability. The qPCR assays using BD multi-species primers developed for quantifying spores trapped in Australian vineyards for SAR 1205 (Billones-Baaijens *et al.* 2018) were suitable for quantifying BD pathogens in grapevine wood samples. The vacuum inoculation method was reliable for standardising *N. luteum* infections, resulting in the conidia infecting the basal, middle and apical part of the inoculated canes. The qPCR analyses further differentiated the low, moderate and high infections, while no pathogen was detected in any of the control canes. This inoculation method allowed the production of a large number of canes with low, moderate and high infection of *N. luteum*, which were eventually used to establish the greenhouse and shadehouse experiments. Our experiments represent the first attempt to investigate the effects of water stress on young vines with low, moderate and high infections of a BD pathogen.

No dieback symptoms associated with BD infections were observed for any treatments across three seasons. However, low vigour, poor yield and smaller root mass were consistently observed in vines under a water deficit regime, indicating they were water stressed. While no external dieback symptoms were observed in inoculated vines, internal staining of the wood was observed on all vine trunks two and three years after inoculation with *N. luteum* spores. Visual assessment of the cross-sections of the trunk showed different severity of staining in vines, with greater staining observed in vines inoculated with higher spore concentrations. The re-isolation of *N. luteum* from the sub-samples for each inoculated vine further confirmed that the vacuum inoculation method was successful in infiltrating the pathogen into the canes. *N. luteum* was not recovered from the non-inoculated controls. This indicates that BD pathogens such as *N. luteum* can colonise the wood internally for several years without causing obvious symptoms. In vineyards, symptoms become more obvious when vines increase in age (SAR 1205, Sosnowski *et al*. 2017).

The qPCR analysis from wood samples taken from the original canes that were vacuum-inoculated with different concentrations of *N. luteum* spores consistently detected *N. luteum* DNA despite the lack of external symptoms for both experiments. For the greenhouse experiment, the water deficit regime significantly increased the amount of *N. luteum* DNA for vines exposed to low (300 conidia) or high (30,000 conidia) disease pressure, indicating that water-stress favours pathogen colonisation. For the shadehouse experiment, water deficit also significantly increased the amount of *N. luteum* DNA but only for vines inoculated with a high inoculum concentration. Vines grown outside in the shadehouse were exposed to seasonal rain and extreme summer heat. These weather conditions likely affected the water treatments and may explain the differences in results compared to the greenhouse vines where the environment was relatively stable.

Previous studies also showed that water stress increased the susceptibility of vines to BD pathogens (Amponsah *et al*. 2014; Qiu *et al*. 2016; Galarneau *et al*. 2019). The results from this study and those reported by Amponsah *et al*. (2014), Qiu *et al*. (2016) and Galarneau *et al*. 2019) are contrary to that of Sosnowski *et al*. (2021), who reported no increase in *D. seriata* colonisation in vines under stress with deficit irrigation. However, this study used a natural method of inoculation by applying spore suspensions on pruning wounds in the vineyard and not by vacuum inoculation followed by propagation in pots. Since vacuum inoculation infiltrated the pathogen into the vascular system, the plant response mechanisms were possibly different to those in the pruning wound infection trial. However, Sosnowski *et al.* (2021) used only one level of inoculum, thus, the infection thresholds that result in symptom development were not established.

The pathogen load detected by qPCR from vines inoculated with a low level of spores (300 conidia) was not significantly different from the non-inoculated controls, suggesting that the vines were able to

withstand a low disease pressure when given sufficient water and appropriate care during the three years of the trial. It is possible that the vines have sufficient defense mechanisms to counteract these low levels of *N. luteum* infections. This new knowledge generated from this pilot study is a crucial step in understanding the impact of this pathogen in young vines, amidst concerns of nursery plant materials being implicated in the poor performance of vines during the establishment phase. Amid concerns on climate change, establishment of infection threshold studies under field conditions is vital to further understand how planting materials with some level of disease infection can withstand extreme vineyard conditions. Infection threshold studies for YVD pathogens such as *C. luteo-olivacea*, *Phaeoacremonium* spp. and *Ilyonectria* spp. are also required, since these species are the most dominant YVD pathogens in Australian nurseries, based on the nursery microbiome studies (Section 11.1.3), and little is known with regard to their impact on vineyard establishment in Australia.

Understanding the threshold of infection and the stress conditions that have the potential to trigger disease expression in vineyards will contribute to the development of management strategies to mitigate YVD and BD infections in nurseries and young vineyards. The first year of this study focused primarily on the evaluation of a published inoculation method for standardising different levels of BD infection. The method was found to be suitable for inoculating different inoculum levels in vines without significant impact on plant viability. The qPCR assays developed for the spore trapping studies (SAR 1205) further allowed the accurate quantification of BD infection from inoculated vines. These methods enabled the production of a large number of grapevine canes needed for the establishment of pot experiments and could be adopted for potential future infection threshold experiments in the vineyard.

In summary, this study provided key evidence that water deficit can increase the susceptibility of young vines to *N. luteum* infection, particularly when the vines are under high disease pressure. This study further showed that vines with a low level of infection were able to withstand the disease for at least three seasons when given sufficient water. These results suggest that young vines with low levels of BD infections can withstand the disease if appropriate care is provided.

11.2.5 References

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12 OUTCOMES AND RECOMMENDATIONS

12.1 Climate specific recommendations to minimise infection

Inoculum dispersal

This study is the first to comprehensively investigate the spore release patterns of both ED and BD pathogens in Australian wine growing regions. The spore trapping studies from 2016 to 2021 revealed sporadic spore dispersal that varied between regions, seasons and years. The number of samples with detection of ED pathogens was higher than that of BD pathogens in most regions. In most cases, spores were primarily detected in late autumn to early spring while spores were also trapped over summer on a few occasions. Spores were generally detected during or within 48 hours of rain but not all rain events resulted in spore detection. The main outcome of this study showed rainfall as a primary factor that triggers the release of either ED or BD spores. At all spore trapping sites, spores were generally detected during or immediately after the occurrence of rain, with as little as 0.2 mm of rain resulting in spore release. Occasionally, spores were continuously detected for up to 14 days, which also coincided with continuous rain. However, not all rain events resulted in spore release, with no spores being detected over several periods despite continuous rain occurring. On some occasions, however, spores were also trapped several days after a rain event, particularly for BD pathogen spores. The detection of spores in the absence of rain suggests that other climatic factors such as relative humidity or dew point may also play a role in the release of these spores.

It is well known that spores of *E. lata* and its closely related species can travel long distances, potentially up to 160 km. However, previous reports suggested BD pathogen spores only spread in rain-splash, up to a few metres, but the detection of BD pathogen spores in a trap located 20 m from the closest inoculum source constitutes the first report of BD pathogen spores becoming airborne for these distances. Spores of BD pathogens were often detected throughout spring and summer, so any wounds created as part of canopy management (i.e. sucker removal, shoot trimming) may also be at risk of becoming infected. Future research will focus on the risk of infection during these activities.

DNA sequencing identified seven species of ED pathogens, with *E. lata* being present and prevalent in most regions. However, *Cryptovalsa ampelina* and a newly reported species, *Diatrype stigma*, outnumbered *E. lata* in two other SA regions. *Eutypella microtheca* and *Eu. citricola* were most prevalent in two New South Wales (NSW) regions, while rarely being detected in SA. This study represents the first report of *D. stigma* and *Cryptosphaeria multicontinentalis* in Australian vineyards. It is likely that these species have been present in Australia without prior identification. Future research to identify different Botryosphaeriaceae species from spore tapes by DNA sequencing is also essential to further elucidate their prevalence and diversity in different wine growing regions in Australia.

Future spore trapping studies will focus on other wine regions of Victoria, Tasmania and Western Australia, and further elucidate the critical times of the year when ED and BD spores are abundant in vineyards in these regions. This will provide localised data for each region that will assist growers in making decisions for optimal pruning time and fungicide treatments of their vineyards.

The rotorod spore traps were able to detect spores of ED and BD pathogens, with ED pathogens predominant in the vineyards located in Urrbrae and Barossa Valley. Pathogen spores of ED were detected at five positions in and around a Shiraz vineyard with substantial dieback symptoms. An increasing gradient of spore numbers was detected from traps located in the north-east corner to the greatest numbers detected in traps at the southern edge of the vineyard, which was consistent with wind recorded predominantly coming from the north during the experiment.

Spores of ED pathogens were detected at all heights tested from 40-250 cm. The greatest number of spores was generally detected at the height of 110 cm, which represented the height of the pruning wound zone of the cordon-pruned vines. ED spores were often also detected at 40 cm, although at lower numbers, which represents the height of the air intake orifice of the Burkard volumetric spore trap. This suggests that data from the Burkard spore traps is representative but may underestimate the number of spores present at the height of the cordon pruning wound zone. Durations of spore collection in rotorod traps from 1 to 23 h were evaluated, with the greatest number of ED pathogen spores detected when the traps were run for 23 h.

In an experiment evaluating spore release from remediated vines in the Barossa Valley, both ED and BD pathogens were detected in much greater numbers in the control vines, compared to the remediated

vines. Visual inspection of the control vines revealed dead cordons with cankers that would likely harbour fruiting bodies of both pathogens, providing a source of new inoculum each time rainfall occurs. Although remedial surgery may reduce the level of inoculum and infection risk within the vineyard, annual wound protection is still necessary to prevent infection from spores derived from inoculum sources outside of the vineyard. Rotorod spore traps provide an effective research tool for monitoring spore dispersal in vineyards under different management regimes.

Updated knowledge on optimum pruning times

The field trial in the Adelaide Hills revealed that wounds were highly susceptible to *E. lata* and *D. seriata* immediately following pruning, after which the susceptibility decreased rapidly over the following 14 days. From 21 days post-pruning, susceptibility was generally negligible. This indicates that wounds are most vulnerable to infection for the first 14 days following pruning, supporting results from a previous trial in McLaren Vale.

For *E. lata*, wounds were generally most susceptible to infection when pruned in early June and least susceptible when pruned in late August. Therefore, in the Adelaide Hills, there may be an advantage in delaying pruning to later in the dormant season to minimise the risk of infection by *E. lata*. These results reflect those of the previous trial conducted in McLaren Vale, where wounds were most susceptible to infection by *E. lata* early in the pruning season.

Susceptibility of wounds to *D. seriata* did not vary greatly between pruning times, so there appears to be no real advantage in avoiding the early pruning time. However, there was minimal natural infection of pruning wounds by *D. seriata*, indicating a low presence of this pathogen in this region. Future research should focus on the extremes, cool and hot-wet climate regions, in order to ascertain whether the period of greatest wound susceptibility varies from that of the more moderate climate regions.

With regard to wound protection, fungicide timing trials indicated that ED and BD pathogens are controlled when wounds are treated with fungicide up to six days after infection, and will continue to provide control of both pathogens for 1-2 weeks. Therefore, a single application of a registered fungicide could provide up to three weeks of wound protection, which covers the most susceptible period of 14 days post-pruning.

In the Adelaide Hills trial, *Cryptovalsa ampelina* was recovered from cane samples with high frequency. Freshly cut canes were free of *C. ampelina*, indicating that infection occurred post-pruning, and the prevalence of *C. ampelina* fruiting bodies on dead arms in the vineyard suggested that this species may play a greater role in ED than previously thought. The increased prevalence of *C. ampelina* in Australian vineyards requires further investigation. Firstly, it will be important to determine how widespread the species is across Australian growing regions. The pathogenicity of *C. ampelina* alone or together with *E. lata* and Botryosphaeriaceae species as part of a disease complex needs to be better understood. Further evaluation of wound protection treatments in the vineyard is also required, to confirm efficacy of currently recommended paints, fungicides and biocontrols.

12.2 Understanding risk of infection during summer pruning

Monitoring spore dispersal using Burkard spore traps revealed that ED and BD pathogen spores were primarily detected in late autumn to early spring, particularly in temperate environments, but spore detections often persisted throughout spring and summer, generally in association with rain, and particularly in sub-tropical environments. This knowledge has raised questions about the risk of infection during shoot trimming and sucker removal practices for canopy management.

A preliminary investigation of wound infection during spring shoot thinning has revealed that the two common wound types, pruning wounds caused during trimming of green shoots and socket wounds caused by the removal of green shoots at their base, were susceptible to infection by both *E. lata* and *D. seriata*. This result confirms previous reports regarding BD pathogens but constitutes the first known report of spore infection of shoot thinning wounds by *E. lata*. With these results, and the reports of spore detection of ED and BD pathogens throughout spring, it is now important to determine the risk of infection in the vineyard following shoot thinning activities.

12.3 Assessment of suitability of LAMP to detect GTD pathogens

Loop mediated isothermal amplification (LAMP) assays were developed and evaluated for their suitability and sensitivity to detect the ED and BD pathogens in spore trap samples and infected plant material. Two LAMP genus-specific primers were designed for BD pathogens belonging to the genus *Diplodia* and *Neofusicoccum*, and five species-specific primers designed for ED pathogens, *C. ampelina, E. lata*, *E. leptoplaca, Eu. citricola, and Eu. microtheca*. Primer sets were suitable for real-time LAMP assays using the Genie II instrument (Optigene, UK), which is lightweight with an internal rechargeable battery, and is ideal for use outside the laboratory.

A rapid DNA extraction protocol that can be efficiently used to extract crude DNA from plants and spore tape samples was developed. This method is easy to use, does not require special storage conditions and can be used in the field but at a lower cost compared to the commercial kits.

Real-time LAMP assays were evaluated for their performance relative to qPCR and fungal isolations. For G-LAMP, the two BD genus-specific primers were able to detect low levels of *N. luteum* and *D. seriata* genomic DNA in vacuum-inoculated vines, indicating that these assays can be used as a rapid diagnostic tool for infected plant material. The *E. lata*-specific primers also detected *E. lata* DNA from vineyard canes inoculated with *E. lata* spores. The real-time LAMP assay was at least twice as sensitive at detecting *E. lata* from inoculated wood compared to fungal isolations. For C-LAMP, positive detection of crude *E. lata* DNA from inoculated canes was slightly higher compared to fungal isolations. Thus, C-LAMP assays can potentially be used for the assessment of cane samples from vineyard trials in place of laborious fungal isolations. The G-LAMP was found to be superior to C-LAMP, thus, it is suited for testing where sensitivity is paramount, such as commercial diagnostic services. However, C-LAMP is a cheaper, viable alternative for fungal isolations and can be used in limited resource laboratories and on-site testing. Performance evaluation of C-LAMP for the detection of other GTD pathogens from infected plant materials will be incorporated into future research to determine its suitability for on-site testing.

Preliminary tests using the two BD genus-specific primers and *E. lata* species-specific primers also indicate that these primers were suitable for colorimetric LAMP. Positive amplification can be assessed visually based on a colour change in the reaction tubes. Unlike real-time LAMP, colorimetric LAMP does not require expensive equipment and can be performed using simple heating equipment. However, evaluation of their performance for rapid pathogen detection from infected plant materials gave highly variable results between replicates and assays. Further studies are required to identify rapid DNA extraction methods suitable for colorimetric LAMP before this can be used for ED and BD field-based diagnostics.

Integration of LAMP assays with a spore trapping system such as the Burkard multi-vial cyclone trap can be further explored in future research aimed at real-time and automatic detection of ED and BD spores in the field, as the real-time technology is developed.

12.4 Optimised wound protection techniques

Information on the effect of adjuvants on wound coverage and fungicide efficacy

Results from the wound spray coverage trial indicated that there was no benefit in adding adjuvants (wetters or stickers) to increase coverage and improve the efficacy of fungicides.

A low water rate (200 L/ha) was intentionally used to simulate sub-optimal coverage of pruning wounds, to test the ability of adjuvants to increase coverage and efficacy. This led to poor disease control, even with the application of two registered fungicides, reiterating the importance of applying the recommended minimum water rate of 600 L/ha to achieve sufficient coverage.

Exposure of water sensitive papers (WSPs) resulted in more than twice the coverage compared to the fluorescent pigment deposited on pruning wounds from the same treatment. Although relative coverage can be more easily estimated using WSPs, it is important to recognise that actual coverage of the wounds may be less than indicated. The development of a protocol for using fluorescent pigment to monitor spray coverage on pruning wounds may be a useful tool for future research and could be used by operators to adjust and calibrate sprayers for optimal spray coverage.

Knowledge on critical timing of biocontrol and efficacy of alternative products

A vineyard trial was established to evaluate the timing of application of Vinevax (*Trichoderma harzianum*) as a wound protectant, followed by inoculation with trunk disease pathogens from 0-28 days. The results showed that application of Vinevax to wounds led to substantial colonisation of *T. harzianum* in the canes. However, it did not provide adequate protection from infection by ED and BD pathogens, particularly in the first three days after pruning, and was less effective than the registered fungicide Gelseal (tebuconazole). Further investigation is necessary into application of higher *T. harzianum* spore concentrations on wounds to speed up colonisation, evaluating other *Trichoderma* species and products, and screening of local isolates from Australian wine regions.

Alternative products were evaluated for protection of pruning wounds against infection by ED and BD pathogens, in a series of detached cane assays. Botector (*Aureobasidium pullulans*) and chitosan both showed promise for ED, but less so for BD. Botector was subsequently evaluated in the vineyard, and there was no reduction of wound infection by either pathogen. Unlike *Trichoderma*, *A. pullulans* was re-isolated at low incidence from canes, suggesting either poor or temporary colonisation. Again, further investigation is required to evaluate the efficacy of *A. pullulans* when applied at higher concentrations on wounds following pruning. Chitosan products developed for disease management should also be further evaluated, and the search for other alternative organic wound treatments should continue to ensure sustainable management of grapevine trunk disease.

12.5 Optimised remedial surgery techniques

Remedial surgery

Remedial surgery trials were established to scientifically evaluate the technique for the control of BD on both own-rooted and grafted vines. Own-rooted vines were able to recover and produce new shoots following remedial surgery. However, grafted vines had lower shoot production compared to own-rooted vines, suggesting that remedial surgery may be more successful in own-rooted vines than in grafted vines.

Four years after remedial surgery, dieback symptoms had re-emerged on vines, albeit with low severity. The vines were not treated with annual pruning wound protection following remedial surgery, suggesting that new infections may have occurred. Thus, application of pruning wound protectants during the pruning season is vital to ensure the long-term success of remedial surgery for the control of BD.

Remedial surgery has proven to be an effective method for managing ED and for increasing the longevity of vines. The preliminary data from these trials showed that this strategy may also be effective for managing BD. However, remedial surgery was found to be most successful in own-rooted vines. Thus, further work on grafted vines is necessary, and other options for inducing shoot production should be considered.

Watershoot induction

Without intervention, 70-87% of remediated trunks produced watershoots over four years. In a world first, five methods anecdotally reported to improve watershoot growth following remedial surgery, were scientifically evaluated. A low incidence of watershoots emerged from within the treatment area, and the majority of watershoots emerged randomly from other parts of the trunks, indicating that there was no influence from most treatments applied at the time of remedial surgery on the production of shoots. However, grafting increased shoot production by 9-13%.

Further evaluation of grafting to increase regrowth following remedial surgery in the following four years was undertaken. Grafting trunks that had not produced a watershoot significantly increased the incidence of trunks with shoot growth by 6 to 19%.

In the final year of the study, watershoots were retained from the previous year and shoot production was recorded at 87-88%. This was comparable to shoot production with grafting in previous years and supports the strategy of retaining watershoots prior to conducting remedial surgery. Further evaluation of this technique in a randomised and replicated trial is necessary, to confirm its success in improving shoot production.

12.6 Knowledge on the susceptibility of clones and scion/rootstock combination

Visual assessment of symptoms in the SARDI germplasm clonal collection and replicated trials in commercial vineyards indicated that the severity of dieback varied between clones, with greater variation observed in the moderately susceptible, compared to the highly susceptible, cultivars. Although results were confounded by differences in vine age of the various clones, and the lack of repetition in clonal plantings, they highlighted possible tolerance in some clones, based on dieback symptoms.

Evaluation of a selection of clones in replicated experiments using DCAs provided no evidence for any tolerance against *E. lata* or *D. seriata* infection amongst them. There was large variation in the colonisation distance that pathogens were recovered from within clones, which made it impossible to distinguish the mean distances between them. Furthermore, lesion lengths did not differ between clones of the same cultivar, and natural dieback was generally similar to that on canes inoculated with pathogens. Therefore, it is most likely that any differences in dieback observed on vines in the field may be due to other factors such as vine age or variation in the ability of clones to tolerate other causes of dieback, such as water stress or poor soil conditions.

Assessment of rootstock trials revealed variation of dieback symptoms between vines planted on rootstocks and on own roots. In the Chardonnay trials, own-rooted vines had less severe dieback than vines planted on most rootstocks. Cabernet Sauvignon vines varied in dieback severity on rootstocks, with own-rooted vines among those with the least severe dieback symptoms. On the other hand, in two of three trials, Shiraz vines on own roots were recorded with more severe dieback symptoms than those on most rootstocks.

Like the clone evaluations, rootstocks did not appear to confer tolerance on Shiraz scion canes inoculated with *E. lata* or *D. seriata* in a vineyard trial. Again, differences in dieback between vines on different rootstocks or own roots that were observed in the vineyard may be due to other factors such as their adaption to water stress, different soil conditions or soil-borne pathogens such as nematodes.

12.7 Knowledge on infection levels in nursery propagation material

This study investigated the diversity and relative abundance of endophytic microorganisms within nursery vines using high-throughput amplicon next generation sequencing. Microbial communities varied greatly between nurseries, varieties and tissue type. Ramsey had the highest microbial population among rootstocks while microbial populations were similar for Shiraz and Chardonnay. Microbial populations were also higher in the roots than the above ground tissues.

Data indicated that 92% of the fungal genera identified are considered to be environmental fungi, and their importance to viticulture is still unknown, while the remaining genera were associated with YVD, bunch rots or fungal species with biocontrol potential that were classified as beneficial. YVD fungi, including the Petri disease pathogens *C. luteo-olivacaea*, *Phaeoacremonium* spp. and *P. chlamydospora* and the black foot pathogen *Ilyonectria* sp. were the most abundant. The ubiquitous nature of these pathogens concurs with other microbial diversity studies internationally. Some beneficial fungi were also present in the nursery vines, although their relative abundance was lower than that of other fungal groups. The predominant species was *Trichoderma,* which was mostly associated with above-ground tissues. The Glomeromycetes, also known as arbuscular mycorrhizal fungi (AMF), were detected primarily in the roots of own-rooted vines, suggesting that *V. vinifera* roots maybe more efficient in recruiting these fungi, compared to rootstocks. Other species such as *Aureobasidum* sp., *Chaetomium* and *Clonostachys* spp. were also detected, suggesting that young vines were harbouring a wide variety of beneficial fungi. In the case of bacteria, the predominant families were Pseudomonadaceae and Burkholderiaceae, which are well known for their biocontrol potential.

There are also some suggestions in the literature that the fungi associated with Petri disease/Esca complex are mostly endophytes and the symptom expression seen in the field is triggered by biotic and abiotic factors such as water stress and extreme temperatures. Thus, it is possible that these YVD-associated microorganisms are merely part of the whole plant microbiome, however, stress factors may influence their transition from an endophytic to a pathogenic phase. Since there is increasing concern about GTD spread in nurseries, future studies to establish the infection thresholds of these YVD

associated fungi in propagation materials that lead to subsequent disease in the vineyard, is vital.

Short duration hot water treatment (HWT) of 54°C for 5 min is used to eliminate external contamination in nursery vines, based on the Australian Standard for Grapevine Propagation Material. According to the microbiome evaluation, this protocol had no impact on the abundance and diversity of fungal populations in young vines, including YVD pathogens. In contrast, HWT altered the bacterial composition in young vines by favouring the heat-tolerant Bacillaceae, while suppressing Pseudomonadaceae populations. Understanding the effects of stress, potentially attributed to HWT, on the overall total health status of young vines will provide some insights on the importance of this practice in the quality management of plant materials in nurseries.

To our knowledge, this study is the first to investigate the effects of HWT on the diversity of endophytic microorganisms of nursery plants in Australia. Understanding the diversity of endophytic microbiota associated with grapevine plant materials may provide information on the overall health status of young vines and assist nurseries in understanding the roles of these endophytic organisms in the quality and establishment of vines in the vineyard.

12.8 Understanding the impact of infection in propagation material on the establishment and productivity of vineyards

This study investigated infection thresholds of BD pathogens in propagation materials and the effects of water stress on disease expression in young vines. A vacuum inoculation method that can standardise the severity of BD infections in experimental vines was shown to be suitable for infiltrating different concentrations of *N. luteum* spores evenly distributed into dormant canes without significantly impacting plant viability. The method proved to be reliable for standardising inoculations, resulting in the conidia infecting canes, and qPCR analyses confirmed differentiated levels of low, moderate and high infections, while no pathogen was detected in any of the control canes. This inoculation method allowed the production of a large number of canes, which were used to establish greenhouse and shadehouse experiments comparing well-watered and water deficit stress treatments on disease development.

No dieback symptoms associated with BD infections were observed for any treatments across three seasons. However, low vigour, poor yield and smaller root mass were consistently observed in vines under a water deficit regime, indicating that they were water stressed. Internal staining of the wood was observed on all vine trunks, with greater staining observed in vines inoculated with higher spore concentrations. The water deficit regime significantly increased the amount of pathogen, particularly for vines exposed to high disease pressure, indicating that water stress favours pathogen colonisation.

The pathogen load detected in vines inoculated with a low level of spores was not significantly different from the non-inoculated controls, suggesting that the vines were able to withstand a low disease pressure when given sufficient water and appropriate care during the three years of the trial. It is possible that the vines have sufficient defense mechanisms to counteract these low levels of infections. The new knowledge generated from this pilot study is a crucial step in understanding the impact of this pathogen in young vines, amid concerns about nursery plant materials being implicated in the poor performance of vines during the establishment phase. Amid concerns on climate change, establishment of infection threshold studies under field conditions is vital to further understand how planting materials with some level of disease infection can withstand extreme vineyard conditions. Infection threshold studies for YVD pathogens such as *C. luteo-olivacea*, *Phaeoacremonium* spp. and *Ilyonectria* spp. are also required, since these species are the most dominant YVD pathogens in Australian nurseries, and little is known regarding their impact on vineyard establishment in Australia.

Understanding the threshold of infection and the stress conditions that have the potential to trigger disease expression in vineyards will contribute to the development of management strategies to mitigate YVD and BD infections in nurseries and young vineyards. The first year of this study focused primarily on the evaluation of a published inoculation method for standardising different levels of BD infection. The method was found to be suitable for inoculating different inoculum levels in vines without significantly impacting plant viability. The qPCR assays further allowed the accurate quantification of BD infection from inoculated vines. These methods enabled the production of the large number of grapevine canes needed to establish pot experiments and could be adopted for potential future infection threshold experiments in the vineyard. This study provided key evidence that water deficit can increase the susceptibility of young vines to *N. luteum* infection, particularly when the vines are under high disease

pressure. Furthermore, vines with a low level of infection were able to withstand the disease for at least three seasons when given sufficient water.

13 COMMUNICATION

13.1 Publications

13.1.1 Scientific publications

Onetto CA, Sosnowski MR, Van Den Heuvel S and Borneman AR (2022) Population genomics of the grapevine pathogen *Eutypa lata* reveals evidence for population expansion and intraspecific differences in secondary metabolite gene clusters. *PLOS Genetics* (https://doi.org/10.1371/journal.pgen.1010153)

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13.1.2 Conference papers

Ayres M and Sosnowski M (2022) Can adjuvants improve fungicide wound coverage and control of Eutypa dieback? 18th Australian Wine Industry Technical Conference, Adelaide 26-29 June.

Ayres M and Sosnowski M (2022) Susceptibility of pruning wounds to grapevine trunk disease pathogens in the Adelaide Hills. 18th Australian Wine Industry Technical Conference, Adelaide 26-29 June.

Billones-Baaijens R, Liu M, Sosnowski M and Savocchia S (2022) Rapid detection of Eutypa and Botryosphaeria dieback pathogens using loop mediated isothermal amplification (LAMP) assays. 18th Australian Wine Industry Technical Conference, Adelaide 26-29 June.

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Ayres M and Sosnowski M (2021) Can the addition of adjuvants to fungicide improve spray coverage of wounds and control of eutypa dieback? 23rd Australasian Plant Pathology Society Online Conference, 23-26 November 2021, p 184.

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Billones-Baaijens R, Liu M, Stodart, B, Sosnowski M and Savocchia S (2021) Characterisation of the endomicrobiome of grapevine nursery plants in Australia. 23rd Australasian Plant Pathology Society Online Conference, 23-26 November 2021, p 64.

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13.1.3 Book chapter

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13.1.4 Industry journals and articles

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13.2 Presentations & radio/television interviews

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Billones-Baaijens R (2022). DNA-based diagnostic tools for grapevine trunk diseases. Digital AgriFood Summit, Wagga Wagga, NSW, 2 June 2022 (20 attendees)

Sosnowski M (2022) Grapevine trunk disease management. Wine Tasmania – Tassie vines going viral. Webinar, 24 May (13 attendees)

Sosnowski M (2022) Managing grapevine trunk diseases in Australia and New Zealand. Canadian Grapevine Certification Network - Grapevine Trunk Diseases Webinar, 28 January (23 attendees).

Billones-Baaijens R (2022) Grapevine trunk disease management for vineyard longevity. Online workshop: Silverado Sustainable Viticulture Summit 2022, California, USA, 21 January (37 attendees).

Billones-Baaijens R (2021) Grapevine trunk disease management for vineyard longevity. Online workshop: Australian Table Grape Association industry meeting, 27 October (~20 attendees).

Sosnowski M (2021) Managing grapevine trunk diseases in nursery source blocks. Workshop: 'Defining the Quality Assurance Objectives for a National Certified Standard for Grapevine Propagation Material' 20 October (26 attendees).

Billones-Baaijens R (2021) Managing grapevine trunk diseases in the propagation process / Nursery vine microbiome / Loop mediated isothermal DNA amplification (LAMP) for rapid testing of trunk disease pathogens. Workshop: 'Defining the Quality Assurance Objectives for a National Certified Standard for Grapevine Propagation Material' 20 October (26 attendees).

Sosnowski M (2021) Grapevine trunk disease management during pruning. SA Central Viticulture Expo & Trade Fair, Longview Winery, Macclesfield SA, 21 September (60 attendees).

Sosnowski M (2021) Grapevine trunk disease management during pruning. AWRI Webinar - Winter pruning to increase the longevity of vines, 29 July (200 attendees).

Sosnowski M (2021) Latest research on preventing & managing trunk diseases in Barossa vineyards. 2021 Barossa Pruning Expo, Nuriootpa Research Centre, Barossa Valley, 16 June (115 attendees).

Billones-Baaijens, R (2021). Grapevine trunk disease management research update. AgriPark Partners Workshop, Charles Sturt University, Wagga Wagga, NSW, 24 June (24 attendees).

Savocchia S and Billones-Baaijens R (2021). Grapevine trunk disease management research update. Practical Science for Viticulture. Charles Sturt University, Orange, NSW, 8 June (38 attendees).

Sosnowski M (2021) Grapevine trunk disease management research update. Focus Vineyard Day, McLaren Vale Grape Wine & Tourism Association. McLaren Vale Tourist Centre SA, 26 May (15 attendees).

Sosnowski M and Billones Baaijens R (2021) Trunk diseases and how to manage them. Tumbarumba Vignerons Association, Tumbarumba Motel NSW, 13 May (38 attendees).

Sosnowski M and Billones Baaijens R (2021) Managing grapevine trunk disease. Riverina Regional Program, Griffith Research Station NSW, 12 May (28 attendees).

Niem J and Billones-Baaijens R (2019) Television interview – Endophytic bacteria from grapevines: a potential biocontrol of trunk diseases. Aired on WIN News Riverina, Prime 7, Nine News, ABC News. December 2019.

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Sosnowski M (2020) Eutypa dieback survey Limestone Coast 2019. Webinar - Limestone Coast Grape and Wine Council Inc. 4 June (20 attendees/recorded).

Ayres M (2020) Managing grapevine trunk disease. Webinar – Elders National Viticulturist Meeting 3 June (30 attendees).

Sosnowski M (2020) Managing grapevine trunk disease. Webinar - Managing Eutypa in Barossa, Barossa Grape & Wine Association, 21 May (60 attendees/recorded).

Baaijens R (2019) Grapevine trunk disease management for vineyard longevity in diverse climates of Australia. National Wine and Grape Industry Centre Viticulture and Oenology Research to Practise Workshop, Wangaratta, VIC, 6 December (~30 attendees)

Ayres M (2019) Managing grapevine trunk diseases. AWRI Roadshow seminar - McLaren Vale Tourist Centre, McLaren Vale SA, 14 November (24 attendees).

Ayres M (2019) Managing grapevine trunk diseases. AWRI Roadshow seminars – Hotel Canobolas, Orange NSW 15 October (15 attendees), Wingecarribee Shire Council Theatrette, Moss Vale NSW 16 October (23 Attendees) and Quality Hotel Dickson (The Tradies) Speedwell Room, Dickson ACT, 17 October (19 attendees).

Sosnowski M (2019) Trunk disease, what now? 12th Wine Tasmania Field Day "Yield from the soil" Tailrace Centre, Launceston Tasmania. 3 September (60 attendees).

Sosnowski M (2019) Grapevine trunk disease management research update (2019). VitiLink Agronomy Forum. Adelaide Rockford Hotel 23 July (12 attendees)

Ayres M (2019) Grapevine trunk disease management research update. FP Ag Supplies - Langhorne Creek Grape Grower Day. Langhorne Creek Winehouse, 16 July (30 attendees)

Sosnowski MR (2019) Disease Management 101: Grapevine trunk disease control in mature vineyards. Keynote presentation – GTD control special session. 11th International Workshop on Grapevine Trunk Diseases, Penticton, British Columbia, Canada, 7-12 July 2019.

Ayres M (2019) Grapevine trunk disease management research update. Clare Valley Wine & Grape Association – Eutypa dieback workshop. Rising Sun Hotel, Auburn, 10 July (40 attendees)

Billones-Baaijens (2019). Grapevine trunk disease management for vineyard longevity in Australia. CSU Viticulture 211 Residential School Guest Lecture. 29 June 2019. (~35 students)

Billones-Baaijens R (2019). Post-pruning and reworking management options for reducing trunk disease infection and spread. AWRI webinar, 13 June 2019 (160 registrants)

Sosnowski M (2019) Grapevine trunk disease management research update (2019). Barossa Pruning Expo – Preventing and Managing Eutypa. Nuriootpa Research Station, 5 June (120 attendees)

Billones-Baaijens (2019). Grapevine trunk disease management for vineyard longevity. AWRI 2019 Roadshow seminar - Mudgee and Hunter Valley, NSW, 29-30 May 2019. (~70 attendees)

Sosnowski M (2019) Grapevine trunk disease management and pruning demonstration. Wines of Western Australia workshops, 28 May, Mt Barker (13 attendees) and 29 May, Margaret River (35 attendees), WA.

Sosnowski M (2019) Grapevine trunk disease research update (Skype presentation). Limestone Coast Grape and Wine Council, Technical Sub-committee meeting. 23 May, Wynns Vineyard Office, Coonawarra.

Billones-Baaijens (2019). Grapevine trunk disease management for vineyard longevity in Australia. NWGIC Board Meeting 9 April 2019. (~10 attendees)

Sosnowski M (2019) Managing grapevine trunk diseases. AWRI Roadshow seminar – Queensland College of Wine Tourism, Stanthorpe, Qld 21 February (19 attendees)

Savocchia S, Billones-Baaijens (2018) Television interview – Grapevine trunk diseases. Aired on WIN News Riverina. 10 Sept 2018.

Anonymous (2018) Recognised in wine awards. News article in The Rural. 15 Nov 2018.

Sosnowski M and Ayres M (2018) Results from commercial remedial surgery trials and GTD research update. Eutypa vineyard walk. The Adelaide Hills Wine Grape and Environment Committee. Nepenthe Vineyard, Hahndorf, 27 November 2018 (29 attendees).

Billones-Baaijens R (2018) Grapevine trunk disease management for vineyard longevity. NSW DPI Pruning Workshop, 13 June 2018, Hunter Valley, NSW (~75 attendees) and 15 July 2018, Orange, NSW (~50 attendees).

Sosnowski M (2018) Managing grapevine trunk disease for vineyard longevity. Viticulture Innovation Day - Pruning for Vine Health and Productivity, 2 July 2018, McLaren Vale (25 attendees) and 3 July 2018, Langhorne Creek (30 attendees).

Ayres M and Sosnowski M (2018) Practical strategies for management of grapevine trunk diseases. SARDI Seminar Series, Plant Research Centre, Urrbrae, 24 May 2018 (30 attendees)

Sosnowski M (2018) Grapevine trunk disease research, Collaborating with McLaren Vale Grape, Wine and Tourism Association since 2003. Wine Australia Board Meeting, Angoves Cellar Door, 18 April (20 attendees).

Ayres M and Sosnowski M (2017) Practical management of grapevine trunk disease. Taylors Wines Toolbox Meeting, Auburn, Clare Valley, 21 November 2017 (18 attendees).

Billones-Baaijens R. (2017) Practical management of grapevine trunk diseases in Australian vineyards. Presentation to staff and students at Lincoln University, New Zealand on 13 November (18 attendees).

Ayres MR, Billones-Baaijens R, Savocchia S, Scott ES and Sosnowski MR (2017) Practical Strategies for Management of Grapevine Trunk Diseases. Crush – The grape and wine science symposium, Plant Research Centre, Waite Campus, 13-14 November 2017 (149 attendees).

Savocchia S and Billones-Baaijens R (2017) Bortryosphaeria dieback in grapevines. Pre-workshop Botryosphaeriaceae *Menace*, 25 Sept; *Science Protecting Plant Health 2017,* Brisbane, QLD, 26-28 Sept. (~50 attendees).

De Garis K, Balnaves P, Kidman C and Sosnowski MR (2017) Using tractor mounted optical sensors to assess grapevine canopy decline. ASVO Mildura Seminar 2017, Intelligent systems – profitable wine growing, Mildura Arts Centre, 2-3 August 2017 (300 attendees).

Sosnowski M and Ayres M (2017) Management of grapevine trunk diseases in Australia and New Zealand. Presentation to 22 staff and students at the French National Institute for Agricultural Research (INRA) in Bordeaux, France on 10 August.

Sosnowski M and Ayres M (2017) Management of grapevine trunk diseases in Australia and New Zealand. WINETWORK industry workshop on Grapevine Trunk Disease and Flavescence Dorée, Santiago de Compostale, Galicia, Spain on 13 August (63 attendees).

Sosnowski M (2017) Practical management of grapevine trunk disease. Victorian Trunk Disease Workshop Series, Mornington Peninsula, Yarra Valley, Milawa and Great Western, 2-4 May. (Presentation delivered by Mark Krstic AWRI with questions covered by Mark Sosnowski by phone link up)

Sosnowski M (2017) New information and recommendations for effective management of grapevine trunk diseases. 2017 Barossa Pruning Expo, Nuriootpa Research Station, 6 June (160 attendees).

Sosnowski M (2017) Grapevine trunk disease management for vineyard longevity in diverse climates of Australia. Limestone Coast Grape and Wine Council Seminar, Coonawarra Hall, SA, 22 March (50 attendees).

14 STAFF

Project leader/supervisor	Mark Sosnowski
Project supervisor	Sandra Savocchia
Principal investigators	Matthew Ayres & Regina Billones Baaijens
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NWGIC technical assistance	Meifang Liu, Richard Baaijens, Aude Gourieroux, Di Hubbard, Jeb Jackson, Helen Pan

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