Australian Government

Australian Grape and Wine Authority

SARDI

SOUTH AUSTRALIAN RESEARCH AND DEVELOPMENT INSTITUTE

Government of South Australia

Primary Industries and Resources SA

NATIONAL Wine & Grape INDUSTRY CENTRE

THE UNIVERSITY OF ADELAIDE AUSTRALIA

Practical management of grapevine trunk diseases



FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **SAR 1205**

Principal Investigator: **Mark Sosnowski**

Research Organisation: **SARDI**

Date: **February 2017**

Project Number: SAR 1205

Project Title: Practical management of grapevine trunk diseases

Principal Investigator: Dr Mark Sosnowski
South Australian Research and Development Institute
GPO Box 397, Adelaide, South Australia 5001
Phone: (08) 8303 9489
Email: mark.sosnowski@sa.gov.au

Chief Investigators: Dr Sandra Savocchia (NWGIC)
Matthew Ayres (SARDI)
Dr Regina Billones-Baaijens (NWGIC)

DISCLAIMER

The authors warrant that they have taken all reasonable care in producing this report. The report has been through the SARDI Sustainable Systems internal review process, and has been formally approved for release by the Chief, Sustainable Systems. Although all reasonable efforts have been made to ensure quality, SARDI Sustainable Systems does not warrant that the information in this report is free from errors or omissions. SARDI Sustainable Systems does not accept any liability for the contents of the report or for any consequences arising from its use or any reliance placed upon it.

© 2017 SARDI

This work is copyright. Apart from any use as permitted under the Copyright Act 1968, no part may be reproduced by any process without prior written permission from the authors.

Table of Contents

1 ABSTRACT 1
2 EXECUTIVE SUMMARY 1
3 BACKGROUND 4
4 PROJECT AIMS AND PERFORMANCE TARGETS 7
5 PRELIMINARY EXPERIMENTS 8
5.1 Botryosphaeria dieback inoculum 8
5.2 Infection potential of *N. luteum* spores 9
6 INOCULUM DISPERSAL 10
6.1 Introduction 10
6.2 Methods 11
6.2.1 Spore trapping 11
6.2.2 PCR primer and probe development 11
6.2.3 Fungal isolates 11
6.2.4 DNA extraction from pure cultures 13
6.2.5 DNA extraction from spore tapes 13
6.2.6 Primer optimisation and sensitivity by conventional PCR 14
6.2.7 Primer validation 15
6.2.8 Quantitative Real-Time PCR (qPCR) 15
6.2.9 Analyses of vineyard spore trap samples 17
6.3 Results 18
6.3.1 Primer development and optimisation 18
6.3.2 Development and optimisation of quantitative real time PCR (qPCR) 23
6.3.3 Spore trapping in vineyards 26
6.4 Discussion 32
6.4.1 Development of molecular tools to detect and quantify ED and BD spores 32
6.4.2 Spore trapping in Australian vineyards 33
7 PRUNING WOUND SUSCEPTIBILITY 37
7.1 Introduction 37
7.2 Effect of pruning time 37
7.2.1 Methods 37
7.2.2 Results 39
7.3 Cultivar variation 45
7.3.1 Methods 45
7.3.2 Results 46
7.4 Discussion 47
8 PRUNING WOUND PROTECTION 49
8.1 Introduction 49
8.2 Timing of application 49
8.2.1 Methods 49
8.2.2 Results 50
8.3 Alternative products 58
8.3.1 Methods 58
8.3.2 Results 58
8.4 Discussion 61
9 REMEDIAL SURGERY 63
9.1 Introduction 63
9.2 Methods 63
9.1 Results 64
9.2.1 Disease severity pre-remedial surgery 64
9.2.2 Shoot production 65
9.2.3 Disease severity post-remedial surgery 67
9.3 Discussion 68

10 DISEASE SUSCEPTIBILITY 69
10.1 Introduction 69
10.2 Methods 69
10.2.1 Cultivar susceptibility 69
10.2.2 Clonal susceptibility 72
10.2.3 Rootstock susceptibility 72
10.3 Results 72
10.3.1 Cultivar susceptibility 72
10.3.2 Clonal susceptibility 84
10.3.3 Rootstock susceptibility 86
10.4 Discussion 88
11 WATER STRESS 90
11.1 Introduction 90
11.2 Methods 90
11.3 Results 92
11.3.1 Riverland trial 92
11.3.2 Barossa Valley trial 95
11.4 Discussion 98
12 OUTCOMES AND RECOMMENDATIONS 100
12.1 Objective 1: Ascertain inoculum dispersal throughout the pruning season 100
12.2 Objective 2: Investigate duration of pruning wound susceptibility 102
12.3 Objective 3: Optimise timing of wound protection treatments 103
12.4 Objective 4: Evaluate potential of remedial surgery to control botryosphaeria dieback 104
12.5 Objective 5: Identify tolerant or resistant germplasm 105
12.6 Objective 6: Examine the impact of drought and regulated irrigation 107
13 REFERENCES 108
14 COMMUNICATION 113
14.1 Publications 113
14.1.1 Scientific publications 113
14.1.2 Conference papers 113
14.1.3 Book chapter 114
14.1.4 Industry journals and articles 115
14.2 Presentations & radio/television interviews 116
15 STAFF 121
16 ACKNOWLEDGEMENTS 121

1 ABSTRACT

Practical strategies for management of the grapevine trunk diseases eutypa and botryosphaeria dieback have been enhanced by new knowledge gained in this project. DNA-based molecular tools were developed to detect inoculum from spore traps, and showed that spore dispersal patterns vary in regions with different climates, highlighting the role of rain and the presence of inoculum over the entire year. Pruning wounds were highly susceptible to infection for the first two weeks, regardless of the pruning time, with susceptibility decreasing thereafter, at various rates, depending on the pathogen. The application of the fungicides Cabrio, Emblem and Folicur to pruning wounds within six days of infection, controlled trunk disease pathogens for a total period of up to three weeks. In the short-term, remedial surgery was successful for control of botryosphaeria dieback on own-rooted vines, with further work required to improve regeneration of scion material on reworked grafted vines. Cultivar susceptibility to dieback varies, with potential for tolerance identified in some germplasm, and preliminary evidence of reduced susceptibility in some clones and rootstocks warrants further investigation. Increased water stress generally decreased the susceptibility of canes to colonisation by trunk disease pathogens, suggesting that drought and deficit irrigation practices are not likely to contribute to an increased prevalence of grapevine trunk disease in Australian vineyards. These outcomes provide new information that is leading to adoption of improved strategies for managing trunk diseases, which will increase vineyard longevity in diverse climates of Australia.

2 EXECUTIVE SUMMARY

The grapevine trunk diseases eutypa dieback (ED) and botryosphaeria dieback (BD) contribute to grapevine decline worldwide, reducing vineyard productivity and longevity, threating the sustainability of the wine industry which contributes $40 billion per annum to the Australian economy. The causal fungi 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, with funding from Wine Australia and industry, has focussed on developing practical management strategies for grapevine trunk diseases.

Burkard spore traps were deployed in four Australian wine regions and protocols for rapid and highly sensitive DNA-based molecular tools were successfully developed and used to detect and quantify ED and BD pathogens on spore tapes. Spores were released at different times of the year, but the seasonal release and number of spores differed between pathogens and regions. ED spores were generally detected in greater numbers than BD spores, possibly due to the size and mode of discharge of these spores. Rainfall was confirmed as the primary factor that triggers the release of spores, which are disseminated throughout the entire year. Therefore, wounds created during sucker removal and shoot trimming may also be at risk of infection, which will require further research. The prevalence of rain varies seasonally between different climatic regions, and therefore influences the timing of spore release. As little as 0.2 mm of rain initiated spore release and, occasionally, spores were continuously trapped for up to two weeks if rain continued. However, not all rain events resulted in spore release and spores were sometimes detected more than a week after any rainfall event, suggesting that spore release may also be attributed to other environmental factors such as relative humidity, dew and wind speed, which requires further investigation. As more data are generated from this and subsequent projects, localised recommendations will be provided for each region to assist growers in making management decisions for grapevine trunk diseases.

Vineyard trials in McLaren Vale, SA and Wagga Wagga, NSW have provided new information on the timing and duration of wound susceptibility to ED and BD pathogens. 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 suggest that, in these trial locations, there may be little advantage in choosing one pruning time over another, in terms of minimising the risk of infection by the trunk disease pathogens evaluated here. However, results highlight the importance of protecting pruning wounds for

at least two weeks post-pruning. Further research is required to evaluate ED and BD pathogens in other regions to provide localised recommendations for the diverse range of climates in Australia.

The timing of fungicide application was evaluated in the vineyard and showed that Cabrio, Emblem and Folicur exhibited curative properties when applied to pruning wounds for control of ED and BD pathogens six days after pruning and inoculation. Therefore a single application once a week may be sufficient to protect wounds, rather than each day following pruning. All three fungicides provided preventative control of BD pathogens for up to two weeks after application, and *E. lata* for up to a week, after which recovery from inoculated controls was significantly reduced due to natural wound healing, reducing the likelihood of infection. In these trials, very high inoculum spore concentrations led to extremely high disease pressure. It is therefore likely that if spore concentrations more closely reflected those that would occur naturally, the fungicides would provide greater disease control than demonstrated here. Future research should utilise lower spore concentrations, more closely reflecting natural conditions, and will need to consider the range of species that are responsible for grapevine trunk diseases.

Results of the susceptibility trials suggest that the most important period of wound susceptibility to trunk disease pathogens is 2-3 weeks post-pruning. The results of the fungicide timing trials indicate that the three fungicides assessed can control ED and BD pathogens when wounds are treated up to six days after pruning and infection, and will continue to provide control for 1-2 weeks. Therefore, if applied one week after pruning, a single application could provide up to three weeks of wound protection. These results have encouraged greater adoption of wound protection strategies for control of grapevine trunk disease.

Remedial surgery, which has previously been shown to control ED, was evaluated as a curative control strategy for grapevines with BD. 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, which, with no intervention, would eventually lead to the death of vines. To date, no symptoms have been recorded on vines treated with remedial surgery, but vines will continue to be monitored to determine longer term success of the strategy. Future research will further investigate remedial surgery for grafted vines and evaluate novel methods of water shoot induction to attempt to improve success of the technique.

The SARDI germplasm collection located in the Barossa Valley consists of a broad range of *Vitis vinifera* cultivars sourced from around the world. The collection was visually assessed for symptoms of trunk disease, and cultivars with low disease severity were selected for evaluation of disease progression. Petit Verdot and Muscadelle were consistently the least susceptible to both ED and BD pathogens, indicating that they are likely to have the greatest tolerance to infection, with Muscat a Petit Grains Rouge, Ughetta, Pinot Noir, Riesling, Petit Meslier and Melon also showing potential for tolerance. Furthermore the Auxerrois and Green Veltliner were also recorded with low susceptibility, but only in detached cane assays in the greenhouse. There was some correlation between cane width and colonisation, which was consistent with findings from previous research. These traits may be useful markers when selecting for tolerant cultivars or new genotypes.

Visual assessment of trunk disease symptoms in the SARDI clonal collection indicated that the severity of trunk disease varied considerably amongst the clones of Shiraz, Cabernet Sauvignon and Chardonnay. Caution should be taken with interpreting these results as they are based on a single visual assessment and may be influenced by the effect of vine age. However, further investigation is warranted, to determine if certain clones might be recommended for future plantings.

A selection of CSIRO rootstock genotypes were also screened for pathogen colonisation using detached canes, and varied in their susceptibility to colonisation by ED and BD pathogens. Several genotypes of *V. vinifera* (Riesling) x *V. cinerea* genotypes were less susceptible than Shiraz. Further evaluation of these and other genotypes with similar parentage is warranted and may lead to the eventual production of rootstocks with reduced susceptibility to trunk diseases. Based on results from the germplasm collection, it may be necessary to evaluate rootstocks further by inoculating canes on vines in the field or providing a longer incubation period in the detached canes.

Water deficit trials in the Riverland and Barossa Valley regions of South Australia provided varying levels of water stress on grapevines. Inoculating pruning wounds with ED and BD pathogens revealed that increased water stress generally decreased the susceptibility of canes to colonisation, suggesting that drought and deficit irrigation practices are not likely to contribute to an increased prevalence of grapevine trunk disease in vineyards. It was observed that fully irrigated vines were most vigorous and had the thickest canes, and, as the irrigation treatments were reduced, so too was the cane width, providing further evidence that narrower xylem vessels restrict the growth of fungal pathogens. The interaction between rootstock and irrigation suggests rootstocks vary in their ability to cope with the soil water conditions, which may affect their response to infection.

In this project, pathogens were recovered up to 198 mm ahead of staining in one-year-old canes, compared with 80 mm in previous research, therefore, the current recommendation of cutting at least 10 cm beyond any stained wood during remedial surgery may need to be increased to 20 cm to ensure the fungus is removed. However, it is imperative that the distance the pathogen can occur ahead of staining in trunks is also determined. Furthermore, both pathogens were able to colonise canes greater than 20 cm in a year, indicating that further research is necessary to determine the maximum rate of colonisation, to assist with recommendations for pruning practices. There was no correlation between staining and pathogen recovery, casting doubt over the usefulness of extent of staining as an indicator of disease severity.

Extension of project outcomes has been delivered to industry through 37 industry journals and articles, 25 conference papers and more than 79 presentations and media interviews. In addition, project team members have contributed to six scientific publications, a book chapter and fact sheet, during the project duration, and anticipate a further eight scientific publications based on results from the current project. Australia continues to lead research on management of grapevine trunk diseases, attracting much interest from around the world. Therefore, it is important that research continues, to increase vineyard longevity in the diverse climates of Australia.

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 rank 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 $40 billion per annum to the Australian economy. Yield losses of up to 1,500 kg/ha have been reported in Australia for Shiraz vineyards (Wicks and 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). The fungus produces toxic metabolites that are translocated to the foliage, resulting in stunted shoots, distortion and necrosis of leaves, reduced bunch size and uneven ripening (Figure 3.1 c,d&e; Moller and 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). Similar 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-shaped 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 the dispersal of *E. lata* and Botryosphaeriaceae inoculums in vineyards, with no published data available for Australian vineyards. Preliminary spore trapping trials conducted in the Hunter Valley from August 2009 – August 2011 suggested a correlation between the detection of Botryosphaeriaceae and other Diatrypaceae following rainfall (NWG 06/01 Theme 2). Current pruning recommendations to avoid infection by ED and BD pathogens are based on 50-year-old data on apricots (Moller and Carter 1965) and research on apricots and grapes in California (Ramos et al 1975, Petzoldt et al 1983b and Úrbez-Torres *et al*. 2010), north-east USA (Pearson 1980, Trese *et al*. 1980) and South Africa (van Niekerk *et al.* 2011a). Recent conflicting information for grapes in other parts of the world (GWT1113; Sosnowski 2012) highlights the need for local research on inoculum dispersal to provide appropriate recommendations for Australian growers.

Wound susceptibility to trunk pathogens varies during the pruning season and among environments, and can persist up to 12 weeks according to overseas research on apricots and grapevines (Carter and Moller 1967, 1970, Moller and Kasimatis 1980, Petzoldt et al 1981, Trese *et al.* 1982, Munkvold and Marois 1995, Chapuis *et al.* 1998, Serra et al. 2008, Úrbez-Torres and Gubler 2011, van Niekerk *et al.* 2011a). However, data for duration of grapevine wound susceptibility in Australian conditions are lacking.

Pruning wounds have been treated with fungicides, paints, pastes and biocontrol agents to protect wounds from trunk disease pathogens, with varying degrees of success (CRV 03/06S; SAR 06/01, SAR 1001, NWG 06/01 Theme 2, Sosnowski *et al.* 2008, 2013b, Gramaje *et al.* 2012, Pitt *et al*. 2012, Ayres *et al.* 2014). Two paints, one fungicide and a biocontrol are registered for control of ED, but no products are registered for BD. Of the fungicides already registered for use on grapes to control other diseases, tebuconazole and fluazinam are the most effective for dual-control of ED and BD. Progress is being made to register further products in Australia. Pruning wound treatments can be applied

effectively with sprayers (SAR 1001; Sosnowski *et al.* 2013b; Ayres *et al.* 2014). It is now imperative to optimise critical timing and frequency of application of fungicides.

Remedial surgery, where infected wood is removed and vines are reworked from lower shoots in healthy wood, is the only effective method for managing ED-affected vines (SAR 06/01; Sosnowski *et al.* 2011b). However, data on effectiveness against BD, a more aggressive and complex disease, are lacking.

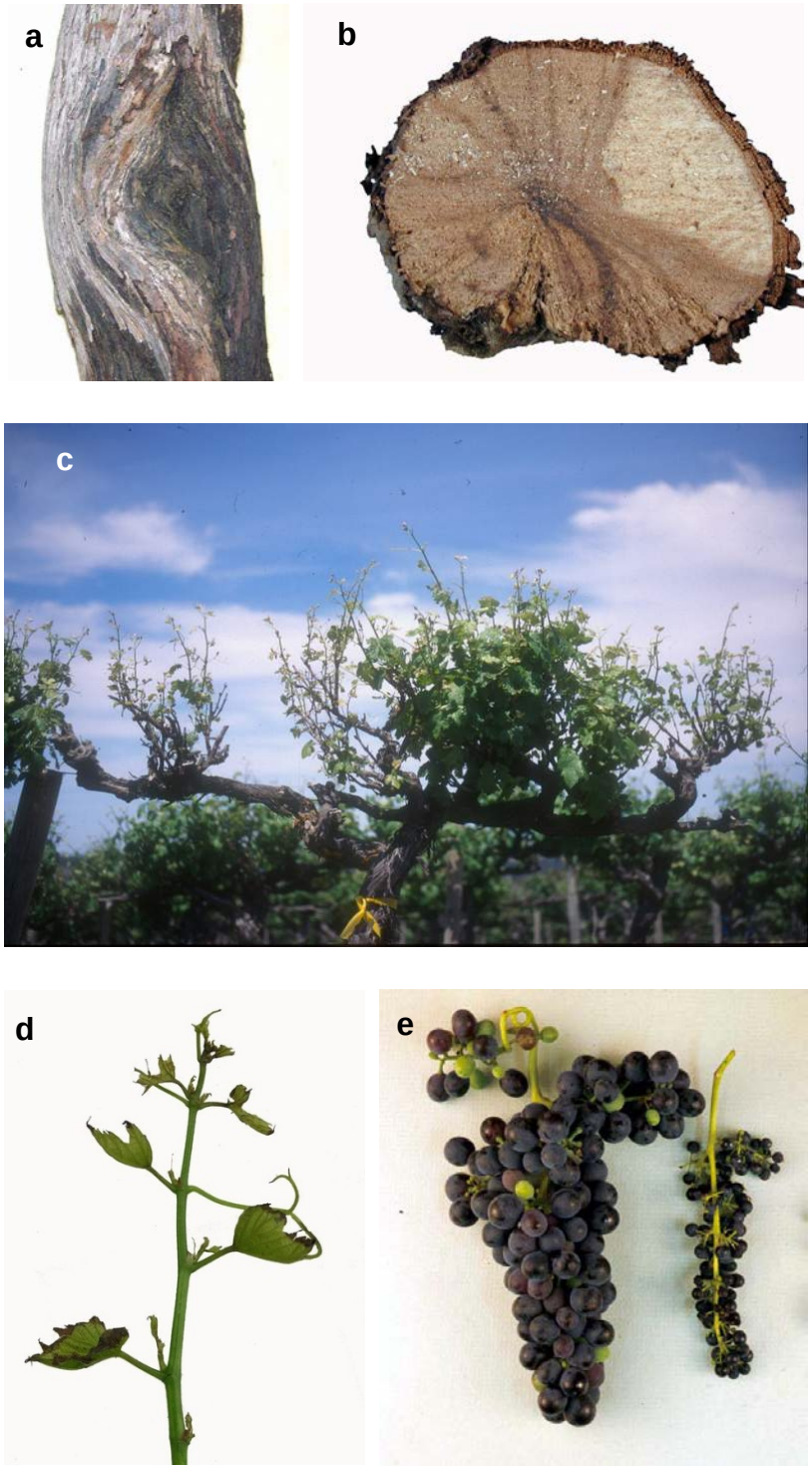


Figure 3.1. Symptoms of eutypa dieback in grapevines. Wood symptoms including (a) external canker and (b) wedge of internal staining. Characteristic foliar symptoms including (c) mature vine displaying stunted shoots, (d) single shoot with cupped leaves with necrotic margins and (e) uneven ripening and reduced bunch size.



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.

There is preliminary evidence of tolerance to trunk disease (Dubos 1987, Peros and Berger 1994, Sosnowski *et al.* 2007b, Travadon *et al*. 2013, Billones-Baaijens *et al*. 2014), which may offer additional means of disease management, however, this requires further investigation. Tolerance to ED has been observed in some *V. vinifera* cultivars and *V. cinerea* x *V. vinifera* (CSIRO) germplasm with resistance to downy mildew (SAR 1001; Sosnowski *et al.* 2013a). Further investigation is required to confirm visual symptom variation amongst *V. vinifera* cultivars, and further evaluation of rootstock material when infected with ED and BD pathogens is also needed.

ED and BD disease severity has been related to climate and stress (CRV 03/06S, SAR 06/01 Sosnowski *et al.* 2007, Sosnowski *et al.* 2011a, van Niekerk *et al*. 2011b, Qiu *et al*. 2014). There have been strong anecdotal claims of increased disease prevalence following drought and Qiu *et al*. (2014) showed a positive correlation between water stressed potted vines and increase in virulence by Botryosphaeriaceae. By elucidating the effect of water stress on susceptibility of field vines to colonisation by ED and BD pathogens, strategies may be developed to mitigate these effects.

4 PROJECT AIMS AND PERFORMANCE TARGETS

Ascertain inoculum dispersal throughout the pruning season

Determine climatic conditions required for spore release by ED and BD pathogens and inoculum dispersal throughout the growing season and, in particular, the pruning season, in different climatic regions of Australia.

Investigate duration of pruning wound susceptibility

Determine the length of time for which wounds on grape cultivars are susceptible to infection by spores of ED and BD pathogens at different times during the pruning season and the link with environmental factors.

Optimise timing of wound protection treatments

Evaluate the preventative and curative properties of fungicides to identify the critical time for application of wound protectants against ED and BD pathogens and seek alternative compounds that can be applied using sprayers.

Evaluate potential of remedial surgery to control botryosphaeria dieback

Remedial surgery techniques developed to manage ED-affected vines will be validated for efficacy on BD-affected vines.

Identify tolerant or resistant germplasm

Evaluate germplasm held by CSIRO and SARDI for response to ED and BD, to identify genetic material that can be incorporated into breeding programs to improve tolerance/resistance to trunk diseases.

Examine the impact of drought and regulated irrigation

Determine the effect of stress caused by decreased water availability due to drought or regulated deficit watering practices on the susceptibility of grapevines to trunk pathogens, which will provide information for improving disease management practices.

5 PRELIMINARY EXPERIMENTS

5.1 Botryosphaeria dieback inoculum

Botryosphaeria dieback, caused by species of the Botryosphaeriaceae, has been the focus of much research over the past decade. However, some results have been hampered by the presence of the pathogen in non-inoculated controls, due to predominance of some species in the environment (Pitt *et al.* 2012). A preliminary experiment was established to compare *Diplodia seriata*, the most commonly found species in Australia with *Neofusicoccum australe*, less prominent in Australia and reported to produce yellow pigment in agar (Pitt *et al.* 2010). A detached cane assay (DCA; Ayres *et al* 2011) was used to compare re-isolation of these species.

Single-node cuttings of Shiraz grapevines were placed in polystyrene boards floating on water, with the top wound surface approximately 1 cm above the node, and the bottom of the canes extending approximately 1 cm below the boards. Wounds were sprayed with sterile distilled water (SDW) and inoculated, using a pipette, with with 1000 spores of *Diplodia seriata* (isolate DAR 79140) or *Neofusicoccum australe* (isolate DAR 79503) suspended in 20 µL SDW. Spores were produced from cultures grown from storage on potato dextrose agar (PDA) under fluorescent lights at 25°C for approximately three months. Spores were washed from the plates and the concentration adjusted to 50,000 spores/mL using a haemocytometer. The trial was set up as a randomised block design with six replications, each replication consisting of three canes. Non-inoculated controls (sprayed with SDW only) were included to assess background infection.

Four weeks later, canes were removed from the boards, and returned to the laboratory for assessment. 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 and then washed twice in SDW. Using sterilised secateurs, and starting immediately below the inoculation point for a distance of 4-5 mm, canes were cut into segments (3 × 2 × 2mm) taken from each side of the margin between live and dead wood tissue. Five wood segments were plated in each plastic Petri dish filled with PDA amended with antibiotic (streptomycin sulfate 100 mg/L), with two plates used for each cane. Samples were incubated on the laboratory bench at approximately 25°C for 3-7 days and then assessed for presence or absence of *D. seriata* or *N. australe* mycelial growth (Figure 5.1). Each species was recovered, with a distinct yellow pigment produced only by *N. australe*. The pigment formed within three days and diminished after six days. No fungal species were recovered from the un-inoculated controls.

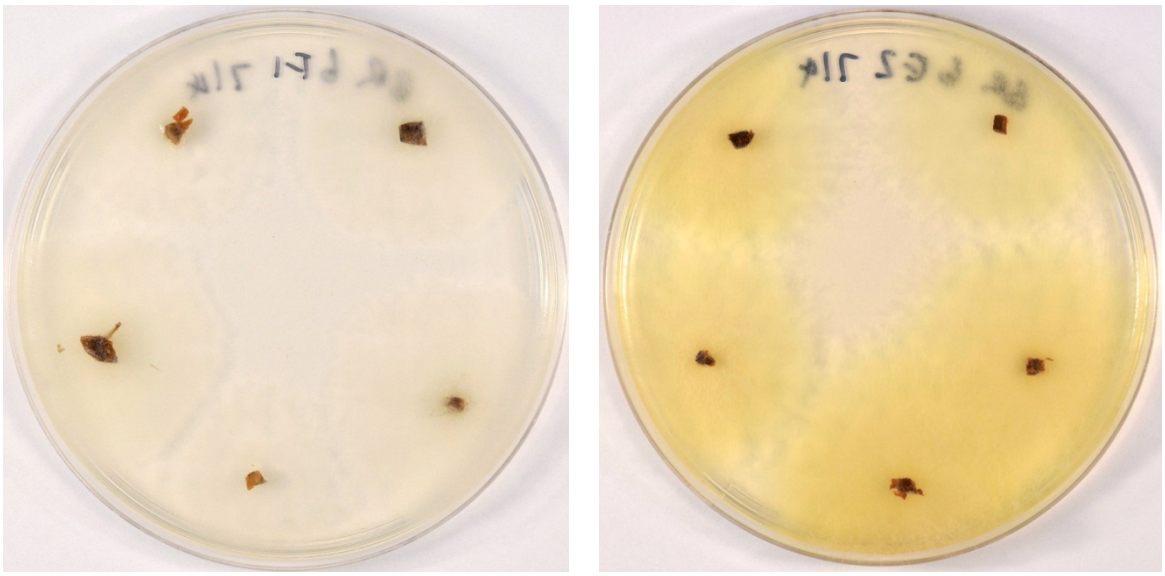


Figure 5.1: *Diplodia seriata* (left) and *Neofusicoccum australe* (right) on PDA three days after plating. The yellow pigment produced by *N. australe* diminished after six days.

5.2 Infection potential of *N. luteum* spores

An inoculum potential experiment was conducted for *N. luteum,* another species producing yellow pigment given that *N. australe* produced very low number of spores in agar. Three isolates of *N. luteum* (B175-2, H12-1 and HH119-1) previously identified by DNA sequencing of the ribosomal RNA gene (Wunderlich *et al*. 2011) were used for this experiment. The three isolates that were stored as mycelial plugs on sterile water were sub-cultured onto fresh PDA amended with chloramphenicol (PDAC, 100 mg/L of agar). Plates were incubated at 25°C overnight in total darkness before being transferred under UV light (Phillips 20w UVB) in a 12h light-dark regime for 4-5 weeks at 18-22°C) until fruiting bodies developed. To harvest the spores, fruiting bodies were scraped off and ground using a sterile mortar and pestle before sieving using two layers of sterile Miracloth (Merck Millipore). The spores recovered from each of the three isolates were combined and spore concentrations were determined using a haemocytometer and concentrations were adjusted accordingly.

One year old dormant canes of Cabernet sauvignon were cut into single node cuttings and placed in polystyrene boards which were floated in plastic tubs filled with tap water. Wounds were inoculated with 1000, 500, 200, 100 and 10 *N. luteum* spores in 10 μL suspensions using sterile micropipettes. The negative control cuttings were inoculated with sterile water. Boards were set up to hold six treatments at eight canes per treatment placed in rows across the boards. The experiments were set up as randomised block designs with four replications (boards). The canes were maintained inside the glasshouse at approximately 27°C. After four weeks, inoculated cuttings were collected to assess for the presence or absence of the yellow-pigmented *N. luteum* colonies. For isolations, the bark was removed and the wood (2-3 cm) was surface-sterilised in 2.5% sodium hypochlorite for three minutes, and rinsed twice with sterile water. The wood were cut into quarters and plated onto PDAC. Plates were incubated at 25°C in total darkness and assessed for *N. luteum* growth from 3-5 days. Data were statistically analysed by analysis of variance (ANOVA) and pairwise comparison using least significant differences (LSD) at 5% significance level.

The incidence of *N. luteum* recovery was similar for the cuttings inoculated with between 200 to 1000 spores of *N. luteum* (93-100%), however incidence recovery decreased when the inoculum concentration was 100 spores (81%) or lower. *N. luteum* was not recovered in any of the non-inoculated controls. These results suggest that under greenhouse conditions, 200 to 1000 *N. luteum* spores have similar infection potential but inoculum lower than 200 spores may result in a lower incidence of infection.

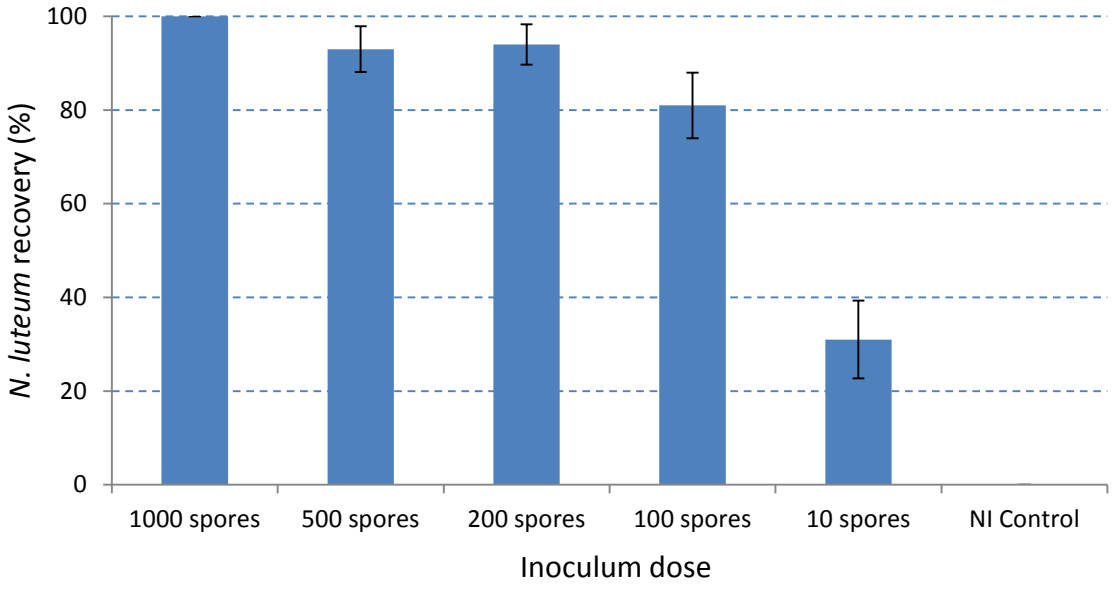


Figure 5.2 Incidence of *Neofusicoccum luteum* recovery from detached canes which were pruned and then inoculated with either 1000, 500, 200, 100 or 10 spores on the day of pruning.

6 INOCULUM DISPERSAL

6.1 Introduction

The spores of eutypa dieback (ED; ascospores) and botryosphaeria dieback (BD; conidia) pathogens are the primary inocula infecting grapevine pruning wounds. These spores are generally dispersed by rain-splash and travel distances depending on wind speed. However, to date there are no published data available on the spore dispersal of these pathogens for Australian vineyards. Current recommendations in Australia are based on information on the dispersal of *E. lata* from apricots published almost 50 years ago (Carter and Moller 1967, 1970). According to research in other countries, the seasonal spore dispersal patterns for ED and BD pathogens from grapevines vary. *Eutypa lata* spore dispersal was reported to be most prominent from autumn, throughout winter and in early spring in California (Ramos *et al*. 1975; Petzoldt *et al*. 1983; Trouillas 2009) and Michigan (Trese *et al.* 1980), during late-winter and early-spring in New York (Pearson 1980) and during late-autumn to mid-winter in South Africa (van Niekerk *et al.* 2010), although summer monitoring was not included in these studies. Botryosphaeria dieback spores were trapped in high numbers in California following rain events primarily during winter (Urbez-Torres *et al*. 2010). However, BD spores were trapped throughout the year, but were most abundant in summer in New Zealand (Amponsah *et al.* 2009), from mid-spring through to mid-autumn in France (Kuntzmann *et al*. 2009) and from late-autumn to early spring in South Africa (van Niekerk *et al.* 2010) and Chile (Valencia *et al*. 2015). These conflicting data from overseas indicate the need for further investigation of the spore dispersal patterns of the ED and BD pathogens in Australia. This will assist in determining the timing of spore release by ED and BD pathogens throughout the growing season and, in particular, during the pruning season in different climatic regions of Australia.

Previous studies on ED and BD spore dispersal from other countries relied primarily on conventional techniques like microscopy and culturing of microorganisms on artificial media, which are extremely time consuming, and are limited in accuracy and sensitivity in detecting these pathogens from environmental samples. Some of these techniques (trapping rainfall and glass slide traps) were also assessed from August 2009 – August 2011 to trap trunk disease organisms in the Hunter Valley however they were found to be time consuming and only yielded semi-quantitative data (NWG 06/01 Theme 2). In recent years, however, molecular techniques have been reported to be useful for detecting pathogens from different environmental samples because they are rapid and more sensitive than conventional techniques. Quantitative real time PCR (qPCR) is now commonly used to detect airborne inoculum of different plant pathogens (Rogers *et al.* 2010; Diguta *et al.* 2010; Duviver, *et al*. 2013) but these methods make use of species-specific primers. Multi-species primers targeting Botryosphaeriaceae species, developed in New Zealand (Ridgway *et al*. 2011) and Italy (Spagnolo *et al*. 2011) were found to be useful in detecting these pathogens from environmental samples but are not specifically-designed for qPCR.

The objective of this study was to trap the spores of trunk disease pathogens from different wine grape growing regions in Australia using rapid and highly sensitive DNA-based molecular tools. This study involved the development of molecular tools, particularly, the design and optimisation of multi-species primers that can detect and quantify ED and BD pathogens from the environment. These primers allow both accurate detection and quantification of ED and BD inoculum from samples collected in Burkard spore-traps deployed in wine regions of Australia with differing climates.

6.2 Methods

6.2.1 Spore trapping

In South Australia, two volumetric spore traps (Burkard Scientific, UK) fitted with battery and solar panel for recharging (Measurement Engineering Australia) were deployed in the Barossa Valley and Coonawarra (Figure 6.1a-b), in August 2013. Two additional spore traps were deployed in Griffith and the Hunter Valley (Figure 6.1c-d), New South Wales in April and May 2014, respectively.

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; Figure 6.1e) using a paintbrush. The drum was placed inside the spore trap chamber and the clock was set for 36 days. In Coonawarra (SA) and Hunter Valley (NSW) the spore traps were placed at the end of a row, while in the Barossa Valley (SA) and Griffith (NSW) the spore traps were placed approximately 20 m from the vines. All spore traps were operated with the orifice 0.5 m above the ground with an airflow of 10 litres per minute. Spore drums were collected and replaced monthly by industry collaborators at each site and sent to the NWGIC for analysis. For processing, the tapes were excised from the drum using fine-point forceps and cut longitudinally into two sections through a Perspex cutting block (Burkard Manufacturing Company; Figure 6.1f) using a sterile scalpel. One section was cut further into 2-day sections (1 × 1.9 cm) using the 36-day rotation cutting block (Burkard Manufacturing Company). The remaining section was stored in an electrical conduit at room temperature in a container with silica gel (BDH, England; SA samples) and at -20ºC (NSW samples) until further use. Each 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 DNA extractions (Figure 6.1f).

Weather data for all sites were sourced from the Bureau of Meteorology (www.bom.gov.au). The weather data for Hunter Valley, NSW were obtained from Cessnock Airport automatic weather station (AWS) which is approximately 300 m from the spore trap while data for Griffith were obtained from Griffith Airport AWS which is approximately 13 km from the site. The weather data for Coonawarra were obtained from AWS 26091, located approximately 300 metres from the spore trap, and the Barossa Valley from AWS 23373, which was co-located with the spore trap.

6.2.2 PCR primer and probe development

Sequences of the ribosomal RNA (rRNA) gene region and β-tubulin gene were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/) for nine Diatrypaceae and nine Botryosphaeriaceae species known to infect grapevines in Australia. The 71 reference sequences for Diatrypaceae species included *E. lata* (17), *E. leptoplaca* (10), *Cryptovalsa ampelina* (7), *C. rabenhorstii* (4), *Eutypella citricola* (12), *Eu. microtheca* (12), *Eu. cryptovalsoidea* (2), *Eu. australiensis* (1) and *Diatrypella vulgaris* (6). The 52 reference sequences for Botryosphaeriaceae species included *Botryosphaeria dothidea* (4), *Diplodia mutila* (5), *D. seriata*, (14), *Dothiorella iberica*, (4), *Lasiodiplodia theobromae* (3), *Neofusicoccum australe* (5), *N. luteum* (5), *N. parvum* (7) and *Spencermartinsia viticola* (4). All sequences were aligned by ClustalW using Mega 4 (Tamura *et al*., 2007) to identify potential binding sites for the target species. All designed primers were tested for their theoretical specificity to their target species using Mega 4 and Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi? LINK_LOC=BlastHome) prior to *in vitro* testing. The primers were further tested for potential secondary structures using mfold (http://www/bioinfo.rpi.edu/applications/mfold). A Taqman probe was further designed for BD qPCR using the Beacon Designer 8 (Premier Biosoft, USA).

6.2.3 Fungal isolates

Nine species of the Diatrypaceae (43 isolates) and Botryosphaeriaceae (51 isolates) from the culture collections of Charles Sturt University (CSU) and Agricultural Scientific Collections Unit (NSW DPI) were used to test the specificity of the different primers at NWGIC (Table 6.1). These isolates were previously identified by partial DNA sequencing of the rRNA and Beta-tubulin genes (Pitt, *et al.* 2010; Trouillas *et. al.* 2011; Wunderlich, *et al.* 2011).

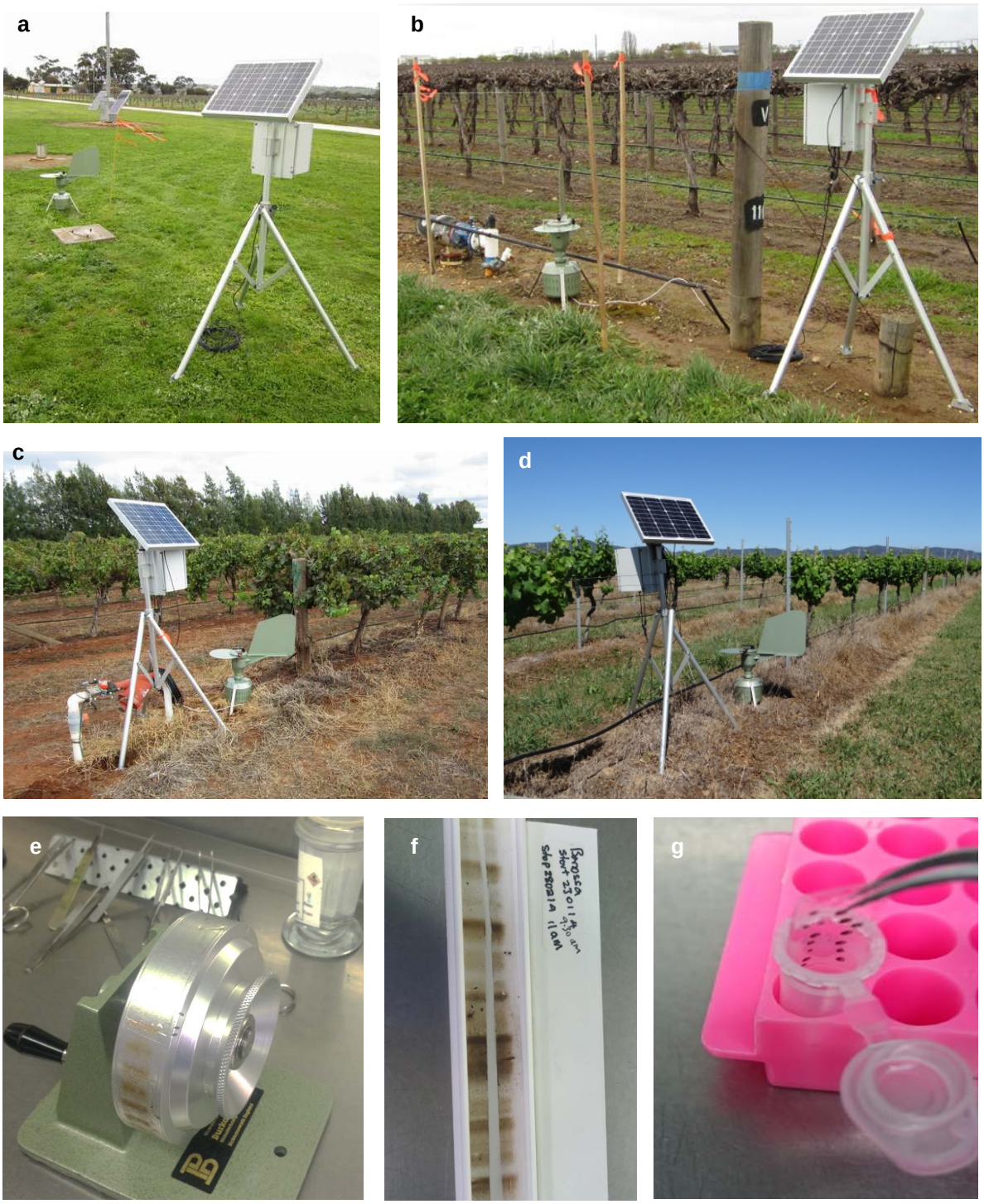


Figure 6.1. Burkard spore traps used for spore-trapping studies and placed within vineyards in South Australia, a) Barossa Valley and b) Coonawarra and New South Wales, c) Griffith and d) Hunter Valley; e) spore drum mounted with Melinex tape; f) excised spore tape stored cut longitudinally prior to analyses; g) spore tape section placed in a tube for DNA extraction.

Ten Diatrypaceae and five Botryosphaeriaceae isolates from Canada, USA and ex-type specimens from the Summerland Research and Development Centre (SuRDC), Agriculture and Agri-Canada, culture collection were further used to test the specificity of the different primers (Table 6.1). These isolates were previously identified by partial DNA sequencing of the rRNA and β-tubulin genes at SuRDC.

From the same CSU and SuRDC culture collections, 57 fungal isolates of the 20 non-target species isolated from grapevines were also used in this study (Table 6.2). The identity of these non-target isolates were confirmed by DNA sequencing of the ITS region of the ribosomal DNA.

6.2.4 DNA extraction from pure cultures

Crude DNA samples from Australian cultures were extracted from the mycelia of all target and non-target species (Table 6.1 and Table 6.2) using PrepMan Ultra® (Applied Biosystems) following the manufacturer's instructions. Except for the biotrophic fungus, *Erysiphe necator*, all isolates were grown in PDA (Oxoid) for 3-7 days at 25°C in total darkness. Mycelia (~100 mg) were scraped from the edge of the colonies for each isolate using a sterile pipette tip and transferred into a sterile 1.5 mL tube containing 100 µL of PrepMan Ultra® preparation reagent. Mycelial suspensions were vortexed for 30 seconds and incubated at 95°C in a heat block for 10 minutes. The tubes were centrifuged for two minutes at 3,220 x g and 50 µL of the supernatant was transferred to a new sterile 1.5 mL tube and stored at -20°C until used for PCR. For *E. necator*, mycelia were scraped directly from infected grapevine leaves using a fine paintbrush and used for extractions as described for pure cultures.

Genomic DNA (gDNA) samples from Australian cultures were further extracted from fungal mycelia for all the Diatrypaceae and Botryosphaeriaceae species (Table 6.1) using both the Isolate II Plant DNA Kit (Bioline, Australia) and the DNeasy Plant Mini Kit (Qiagen, USA). Pure cultures for all species were grown in potato dextrose broth (PDB, Oxoid) at 25°C in total darkness for 3 and 7 days for the Botryosphaeriaceae and Diatrypaceae species, respectively. Mycelia were separated from the broth using a sterile Miracloth (CalBiochem), wrapped in sterile aluminium foil, snap-frozen in liquid nitrogen and stored at -80°C until required. Genomic DNA was extracted from frozen ground mycelium following the manufacturer's instructions. For isolates from SuRDC, gDNA of isolates were extracted using the FastDNA Spin Kit (MP Biomedicals). Pure cultures for all species were grown in PDA for 3-7 days and gDNA was extracted from the aerial mycelia of each isolate following the manufacturer's instructions.

DNA concentrations for all gDNA were determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The concentrations were further verified by loading 5 µL of each gDNA in 1.5% agarose (Bioline) and separated by electrophoresis (10v/cm for 45 cm) immersed in 1× TAE buffer (40 mM Tris acetate, 2 mM Na2EDTA, pH 8.5). The Hyperladder™ 1 Kb (Bioline) and High Mass Ladder (Invitrogen) were run in either the first or last lane of each gel, respectively. The gels were stained using 1× GelRed™ nucleic acid gel stain (Biotium, Hayward, CA) and visualised under UV light using a Gel Doc XR+ Imaging System (BIO-RAD Laboratories, Pty, Ltd.). DNA mass per microlitre of each gDNA sample was calculated using Image Lab 5.1 imaging software (Bio-Rad) using the two mass ladders as references.

6.2.5 DNA extraction from spore tapes

Two published DNA extraction protocols (Rogers *et al.* 2009; Duvivier *et al*. 2013) and two commercial DNA extraction kits (PowerWater® DNA Isolation Kit, MoBio Laboratories; Gentra Puregene, Qiagen) were initially tested for their suitability to extract DNA from spore tapes. The preliminary tests were conducted using the spore tapes (2 × 4.8 cm) coated with Tangle-Trap®. The tapes were artificially inoculated by pipetting 20 µL suspensions of either *N. luteum* or *E. lata* spores of different concentrations (100 to 10,000 spores) and used for DNA extractions at three replicate tapes per sample. To determine the efficiency of the protocol, all samples were tested by standard and nested-PCR described in the next section using the multi-species primers: (a) BOT100F - BOT472R (Ridgway *et al.* 2010); and (b) DITS-1B - DITS-R2, for Botryosphaeriaceae and Diatrypaceae species, respectively. The PCR products were visualised by gel electrophoresis as previously described.

The modified Gentra Puregene protocol that showed high DNA yield and consistent results in the preliminary tests was used for the DNA extractions from vineyard spore tape samples. DNA extractions from the 2-day spore tape sections (1 × 1.9 cm) collected from the four spore trapping sites were conducted using the following protocol. To extract DNA, 500 µL of Cell Lysis solution (Gentra Puregene) and 10 µL of Proteinase K (Promega) were added to a lysing tube containing the 2-day tape sections previously prepared (Section 6.1.3). The tubes were placed into a tissue lyser (Qiagen) microtube adapter and shaken twice for 90 seconds at 30 Hertz with one minute cooling on ice in between each shake. The samples were incubated at 55°C overnight in a water bath. After incubation, tubes were inverted 10 times and 1.5 µL of RNAse A (4 mg/mL, Qiagen) was added, inverted 25 times and incubated at 37°C for 30 min. The tubes were cooled at room temperature and 167 µL of Protein Precipitation solution (Gentra Puregene) was added to the cell lysate and mixed by vortexing at high speed for 20 s. The mixture was centrifuged at 16,000 ×*g* for 3 min and the supernatant (~650 µL) was transferred to a new sterile 1.5 mL tube. The DNA was precipitated by adding 500 µL of ice-cold isopropanol and 1 µL of glycogen (Qiagen) to the supernatant, inverted 30–50 times and centrifuged at 16,000 ×*g* for 1 min. The supernatant was discarded and dabbed dry onto clean absorbent paper. The DNA pellet was washed by adding 300 µL of ice-cold 70% ethanol and the tube was inverted gently (~10 times) and centrifuged for 1 min at 16,000 ×*g*. The ethanol was decanted gently and the tube containing the DNA pellet was air dried for 1-2 hr at room temperature. Finally, the DNA was re-suspended by adding 30 µL of nuclease-free water (Qiagen) and stored at -20°C until required for PCR.

To check any variations in each batch of DNA extractions, three control tapes (1.9 × 1 cm) were included in each batch (~45 samples) of DNA extractions. These were: 1) tape inoculated with ~1000 *N. luteum* conidia; 2) tape inoculated with ~1000 *E. lata* ascospores; and 3) non-inoculated tape. Each control tape was placed inside a 1.5 mL lysing tube previously described and stored at -20°C until required for DNA extractions. One tube each of the control tapes was included in each batch of DNA extraction and PCR.

6.2.6 Primer optimisation and sensitivity by conventional PCR

Prior to qPCR, the optimum annealing temperature for each primer pair was determined using conventional PCR. The PCR was set-up on a gradient block thermal cycler (C100™ Thermal Cycler, Biorad). The 25 µL PCR reactions contained 1× PCR buffer (Bioline), 0.2 µM of each primer (Geneworks), 1.25 U of MyTaq™ DNA Polymerase (Bioline) and 1 µL of either crude or gDNA of three representative species for each pathogen group. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s annealing (gradient run of 54°C to 64°C) and 30 s at 72°C and a final extension of 72°C for 5 minutes. Following PCR amplification, 5 µL of each PCR product were separated by electrophoresis (10 V/cm for 50 min) in a 1% agarose gel (Bioline, Australia) in 1× TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.0). Gels were stained with 1× GelRed™ nucleic acid gel stain (Biotium, Hayward, CA) and visualised under UV light using a Gel Doc XR+ Imaging System (BIO-RAD Laboratories, Pty, Ltd.). The optimum temperatures for each primer were further confirmed using qPCR.

The detection sensitivity for ED primers, DITS1B-DITSR2 and DIA16F-DIA89R was further tested by conventional PCR. The gDNA of *E. lata* (ADSC-1) and *Eu. citricola* (T3R2S2) were serially diluted from 10 ng/µL to 10 fg/µL and 1 µL of each dilution was used as a template using the optimum PCR reaction and thermal cycling condition previously described. The sensitivity of the primers was further assessed using nested-PCR by initially amplifying the serially-diluted template DNA using the DITS-1B and DITS-R2 primers following the same thermal cycling conditions previously described. The products from the first step PCR were diluted 1:100 in nuclease-free water and 1 µL was used as a template for the second step PCR using multi-species primers DIA16F-DIA89R as previously described.

The BD multi-species primers, Bot BetaF1-Bot BetaR1 and Bot100F-Bot472R (Ridgway *et al.* 2010) were also tested for their specificity and sensitivity to their target species using one-step and nested PCR reactions. The thermal cycling protocol described previously was used for testing, but using the universal primers Bt2a and Bt2b (Glass and Donaldson, 1995) and ITS-1 and ITS-4 (White *et al*. 1990), respectively, as the primers for pre-amplification of nested-PCR.

6.2.7 Primer validation

To validate the results obtained from the NWGIC laboratory, all primers were further tested for their specificity at the SuRDC Plant Pathology Laboratory in Summerland, British Columbia, Canada during March-May 2016 using the SuRDC collection, which includes isolates from Australia, Canada, USA and ex-type specimens from CBS-KNAW Culture Collection, The Netherlands (Table 6.1) using the protocols described previously.

6.2.8 Quantitative Real-Time PCR (qPCR)

6.2.8.1 Preparation of standards using gBlocks® Gene Fragments

The 71 reference sequences for Diatrypaceae and 52 reference sequences for Botryosphaeriaceae species were further aligned by ClustalW using Mega 4 (Tamura *et al*., 2007) to design gBlocks® Gene Fragments. The gBlocks® Gene Fragments are single copies of a DNA fragment (in this case the rRNA and β-tubulin gene) that were chemically-synthesised (Integrated DNA Technologies, USA). These double-stranded synthetic DNA fragments with known mass and volume are suitable for use as standards for the ED and BD qPCR. A 460 bp (Diat-5S) and a 410 bp (Bot-Btub) gBlocks® were designed and synthesised for ED and BD qPCR, respectively. The 500 ng gBlocks® were resuspended in 50 µL of TE Buffer (Tris and EDTA, pH 8.0; Sigma Aldrich) following the manufacturer's recommendation to reach a final concentration of 10 ng/µL and used as standards for the ED and BD qPCR standards.

The mass of the Diat-5S and Bot-Btub gBlocks were calculated using the formula adapted from Applied Biosystems (http://www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf).

$$M = [n][1.096e\text{-}21\ g/bp]$$

Where n = gBlock size (bp)
M = mass
e-21 = x 10^{-21}

6.2.8.2 Development of ED qPCR using SYBR green

All qPCRs for ED using multi-species primers Diat16F-Diat89R were performed in 0.1 mL strip tubes and caps (Qiagen) using a RotorGene 6000 (Corbett Life Science, Qiagen). To prepare the standards for ED qPCR, a 10^8 copies/µL standard solution was prepared from the original Diat-5S gBlock solution of 10 ng/µL. A 10-fold dilution from 10^6 to 10^1 was further prepared and used to develop a standard curve using the qPCR conditions described below. Optimum primer concentrations were previously determined by running qPCR using 300 nm, 400 nm and 500 nm concentrations for each primer per reaction. The optimal concentration that resulted in high efficiency (90-110%) with minimal by-products (primer dimers) after 30 cycles was used for the succeeding qPCR. The 20 µL qPCR reaction contained 10 µL of 2× SsoAdv Universal SYBR Green Supermix (Biorad, USA); 500 nm of each primer, 2 µL template and nuclease-free water (Qiagen). The qPCR conditions were set-up using the optimal annealing temperature and primer concentration as determined previously. All qPCR reactions were done using four replicates in three independent assays using the following thermal cycling conditions: 3 mins at 98°C, followed by 35 cycles of 98°C for 30 s, 58°C for 30 s and fluorescence detection at 58°C for 15 s. After the final amplification cycle, a dissociation (melting) curve (72°C to 95°C) was generated to check the specificity of the amplification reaction. For each assay, three controls were included: a) non-template control (NTC, H_2O); b) positive control (*E. lata* gDNA, 2 ng); and c) negative control (*N. parvum* gDNA, 2 ng).

The quantification cycle (Cq) value for each Diat-5S gBlock standard sample was calculated and analysed using the Rotor-Gene Q Series software (Version 2.3.1) to generate a standard curve. The Cq values are defined as the cycle numbers at which detectable fluorescent signal occurs in each qPCR sample. The number of copies for each Diat-5S gBlock standard dilution was plotted against the Cq values and the resulting regression equations were used to quantify the number of copies of each target gene in the unknown samples. The limit of detection (LOD) for the Diat-5S gBlock was determined following the MIQE guidelines (Bustin *et al.* 2009) which was based on the number of copies required to obtain reproducible detection in three consecutive assays.

To determine the number of copies of the target rRNA gene in a haploid genome of Diatrypaceae species, the gDNA of *C. ampelina* (CSU01), *E. lata* (ADS15-C), *E. leptoplaca* (RGA02) and *Eu. citricola* (T3R2S2) that were previously quantified was used to prepare a 10-fold serial dilution from 2 ng to 20 fg. Each gDNA dilution was analysed by qPCR with Diat-5S gBlock standards using four replicates in three independent assays following the conditions previously described. The quantification of target rRNA gene per nanogram of DNA was achieved by comparing the Cq values to each gDNA concentrations with the Cq values of the Diat-5S gBlock standards.

The specificity of the ED primers was tested by qPCR using ~2 ng of gDNA of four Botryosphaeriaceae species: *D. seriata* (A142a), *N. parvum* (B22a), *L. theobromae* (C2), and *S. viticola* (L19). The qPCR was run in four replicates and repeated twice using the protocol described previously. For each assay, three controls were also included: (1) 2 pg Diat-5S gBlock as standard; (2) non-template control (NTC, H_2O); and (3) *E. lata* gDNA (2 ng) for positive control.

To test the effect of non-target DNA on the sensitivity and specificity of the method, ED qPCR was conducted by comparing the quantification of 2 pg of *C. ampelina* DNA (CSU01) alone and when the same DNA was spiked with 200 pg of either *S. viticola* (LCVT8-1) or *L. theobromae* (RA1-4). The assay was run using four replicates and repeated twice. The data were analysed by comparing the Cq values for each sample using one-away ANOVA and the means were separated by LSD at $P \leq 0.05$ using GenStat 17th Edition.

6.2.8.3 Development of BD qPCR using TaqMan probe

All qPCRs for BD using multi-species primers were also conducted in 0.1 mL strip tubes and caps (Qiagen) using a RotorGene 6000 (Corbett Life Science, Qiagen). The BD qPCR primers (Bot BetaF1 and Bot BetaR1) and the multi-species primers (Bot100F – Bot472R) developed by Ridgway *et al*. (2010) were initially tested for their suitability to SYBR green qPCR as described in ED qPCR. Preliminary results showed both multi-species primers produced PCR by-products (primer dimers) when the DNA templates were very low. Since SYBR green dye can attach to any double stranded DNA including primer dimers, this can result in false quantification of non-specific PCR products. To resolve this problem, a Taqman probe (Taq-Bot) for BD primers (Bot BetaF1 and Bot BetaR1) was further developed as described previously. The Taq-Bot probe was labelled at the 5' end with 6-carboxyfluorescein (FAM) and a double-quencher (internal Zen with 3' Iowa Black® FQ; Integrated DNA technologies, USA). This probe uses a different fluorescence chemistry from SYBR green and binds only to the PCR products and not to primer dimers. Preliminary tests showed the BD primer-probe combination was highly specific and can accurately quantify BD pathogen DNA and was used in the subsequent qPCR analyses from spore tape samples.

The Bot-Btub gBlock standards were further prepared and serially diluted 10-fold as previously described for Diat-5S gBlock. The quantification cycle (Cq) value for each Bot-Btub standard sample was also calculated and analysed and used to develop a standard curve as previously described. Ten-fold serial dilutions from 10 ng to 100 fg of gDNA of *D. seriata* (A142) and *N. parvum* (B19) were also prepared and used to determine the number of copies of β-tubulin gene in a Botryosphaeriaceae haploid genome. The Bot-Btub gBlock standard and gDNA dilutions were analysed by qPCR using GoTaq® qPCR Master Mix (Promega). Each 20 µL reaction contained 10 µL of 2× GoTaq® Master Mix; 500 nm of each primer, 250 nm of Taq-Bot probe and 7 µL template. The qPCR conditions were set-up using the optimal annealing temperature and primer concentration determined previously. All qPCR reactions were conducted using the following thermal cycling conditions: 2 mins at 98°C, followed by 45 cycles of 98°C for 10 s, 60°C for 30 s and fluorescence detection at 58°C for 30 s.

To test the sensitivity of the method, qPCR was performed on each dilution as previously described to determine the LOD of the assay. All Bot Btub gBlock standard solutions and gDNA were tested by qPCR using four replicates in three independent assays following the conditions previously described. For each assay, three controls were included: (1) NTC (H_2O); (2) positive control (*N. luteum* gDNA, 7 ng); (3) negative control (*E. lata* gDNA, 3.5 ng).

The BD qPCR was further tested for specificity by running qPCR using ~3 ng of gDNA of four Diatrypaceae species: *C. ampelina* (CSU01), *Eu. citricola* (T3R2S2), *E. lata* (ADS15-C) and *E.*

leptoplaca (RGA02). The qPCR was run using four replicates and repeated twice using the protocol described previously with Bot-Btub gBlock (7 pg) as standard; H_2O for NTC and *D. seriata* gDNA (A142a, ~1 ng) as a positive control.

To test the effect of non-target DNA on the sensitivity and specificity of the method, BD qPCR was conducted by comparing the quantification of 2 pg of *D. seriata* gDNA (DGR2) alone and when the same DNA was spiked with 200 pg of either *E. lata* (ADSC-1) or *C. ampelina* (CSU01). The assay was run using four replicates and repeated twice. The data were statistically analysed as described previously in ED qPCR.

6.2.9 Analyses of vineyard spore trap samples

6.2.9.1 Nested-PCR for vineyard spore trap samples

All DNA extracted from spore trap samples (2-day sections) from the Barossa Valley (August 2013-October 2014); Coonawarra (November 2014- November 2015); Hunter Valley (May 2014-July 2015); and Griffith (May 2014-September 2015) were initially tested for the presence or absence of both ED and BD spores by nested-PCR. To detect ED spores, nested-PCR was conducted by initially amplifying spore tape DNA extracts using the ED multi-species primers DITS1B - DITSR2 (Step 1), followed by DIA16F - DIA89R (Step 2) using the protocol described previously (Section 6.1.4.4) but using 2 µL of DNA as template for each PCR step. To detect BD spores, nested-PCR was conducted by initially amplifying spore tape DNA extracts using the universal primer ITS1F and ITS4 (Step 1) with the annealing temperature of 55°C at 25 cycles. The second step PCR was conducted by running 2 µL of the Step 1 PCR products as template using the primers Bot100F and Bot 472R and the protocol previously described (Ridgway *et al*. 2010). For each nested-PCR, four controls were included: a) NTC (H_2O); b) non-inoculated spore tape; c) *N. luteum* conidia inoculated tape; and d) *E. lata* ascospore inoculated tape. PCR products from the second step of each nested PCR were visualised by electrophoresis following the protocol described in Section 6.1.4.2.

6.2.9.2 ED and BD qPCR of vineyard spore tape samples

Nested-PCRs were further analysed by qPCR to determine the number of spores trapped in each 2-day period for all spore tape samples that tested positive to either or both ED and BD pathogens. Each qPCR was performed following the ED and BD qPCR protocols described previously and the following controls and standards were included: a) Bot-Btub or Diat-5S gBlock standard solutions (7 and 2 pg, respectively); b) H_2O (NTC); c) non-inoculated tape control; and d) *N. luteum* inoculated tape; e) *E. lata* inoculated tapes. All samples were run in duplicate except for the NTC and gBlock standards that were run in four replicates.

To analyse samples for each qPCR run, the standard curves previously developed for ED and BD qPCR were imported in the software. The standards (either Bot-Btub and Diat-5S gBlocks) that were included in each run were used to calibrate the imported standard curve. The mean Cq values for each unknown sample were plotted against the Cq values of the standards and the resulting regression equations were used to quantify the number of spores in each sample as previously described.

6.2.9.3 Data analyses

To calculate the number of ED and BD spores in 2-day spore tape samples, the following formula was used:

$$N = Log_{10}(Q)(D/T)(2)$$

Where:

N	=	Calculated no. of spores in a 2-day period
Q	=	the mean number of spores detected by qPCR
D	=	the DNA volume (30 µL) extracted from a half section of a 2-day sample
T	=	the amount of DNA template (µl/reaction) used in one qPCR reaction.
2	=	the total number of tape sections for the 2-day collection period.

For the ED qPCR, 2 µL DNA was used for each qPCR reaction while 7 µL DNA was used for BD qPCR.

All *N* values were transformed to Log_{10} to reduce the skewness of a large range of values. The N values and its Log_{10} values were plotted with the total rainfall and mean maximum temperature for the same period to determine the correlation between spore release and weather data.

6.3 Results

6.3.1 Primer development and optimisation

6.3.1.1 Primers for conventional PCR

Alignment of the ribosomal RNA gene sequences identified two binding sites and one pair of multi-species primer for conventional PCR was designed. For the Diatrypaceae multi-species primer, the forward primer DITS-1F (AAMCCATGTGAAYTTACCT) was degenerate at nucleotides 3 and 13 and is designed to anneal to the ITS-1 region, while the reverse primer DITS-R2 (CCAAGCARMTRGGGCTTGA) was degenerate at nucleotides 8, 9 and 11 and is designed to anneal to the ITS-2 region of *E. lata*, *E. leptoplaca*, *C. rabenhorstii*, *Eu. citricola*, *Eu. microtheca*, *Eu. cryptovalsoidea* and *Diatrypella vulgaris* but not *C. ampelina* and *Eu. australiensis* due to high sequence dissimilarities for these two species.

The optimum annealing temperature for the multi-species primers DITS-1F/DITS-R2 was 58°C which amplified a single PCR product of approximately 305-350 bp from all seven target species (*E. lata*, *E. leptoplaca*, *C. rabenhorstii*, *Eu. citricola*, *Eu. microtheca*, *Eu. cryptovalsoidea* and *Diatrypella vulgaris)* while none of the *C. ampelina* and *Eu. australiensis* isolates were amplified using these primers (Table 6.1). No PCR products were observed with the nine Botryosphaeriaceae species (51 isolates) or 19 other non-target fungal species (40 isolates) from grapevines, indicating that they are highly specific to the seven target Diatrypaceae species tested (Table 6.1).

6.3.1.2 Sensitivity of Diatrypaceae multi-species primers

The standard PCR using serially diluted gDNA of *E. lata* and *E. leptoplaca* demonstrated that the DITS1B - DITSR2 (Figure 6.2a) could detect ~10 pg of gDNA while the nested-PCR protocol was able to detect as low as 10 fg of gDNA (Figure 6.2b). The DIA16F and DIA89R multi-species primers was also able to detect ~10 pg and 10 fg of gDNA for standard and nested-PCR, respectively (data not shown)

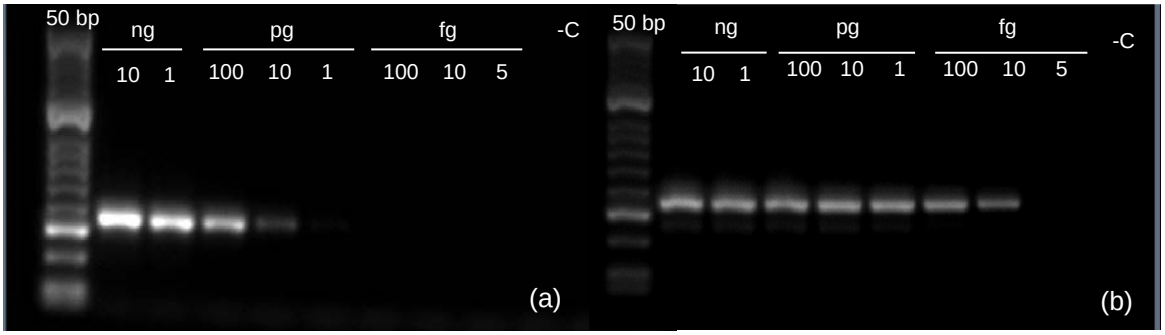


Figure 6.2. 1% agarose gel of PCR products using DITS-1B and DITS-R2 primers. (a) Standard PCR using serially diluted DNA of *E. lata* isolate (OR039) as template; (b) Nested PCR using the diluted (1:200) product of the Step 1 PCR as template. 50 bp = HyperLadder™ 50 bp (Bioline), -C = non-template negative control.

6.3.1.3 Species-specific primers for Eutypa lata and Cryptovalsa ampelina

The alignment of the β-tubulin gene identified four binding sites that are unique for either *E. lata* or *C. ampelina* and these sites were used to design primers specific for these two species. The forward

primer BTEuF1 (ATATCTCCAGTCTTCCTATC) and the reverse primer BTEuR1 (AGCTTAAATCACACAATCAC) were designed to anneal specifically to the Beta-tubulin nuclear gene region of *E. lata* with the expected PCR product of 327 bp. The forward primer CampBTF1 (TACGTAACTGACCTCATTAC) and reverse primer CampBTR1 (TACGGACAGGAGTGTCAGTA) were further designed to specifically anneal to the β-tubulin gene of *C. ampelina* with the expected PCR product of 215 bp.

Amplifications of the species-specific primers BTEuF1 and BTEuR2 and CampBTF1 and CampBTR1 are shown in Table 6.1. All 11 *E. lata* isolates were amplified using the primers BTEuF1 and BTEuR2 while no PCR products were observed with the other non-target species nor the eight other Diatrypaceae species included in this study. All eight *C. ampelina* isolates were further amplified using the CampBTF1 and CampBTR2 primers and no PCR products were observed with the DNA from all non-target or other Diatrypaceae species. These results suggest that both primer pairs are highly specific to their target species.

6.3.1.4 ED qPCR using multi-species primers

For ED qPCR, the Diatrypaceae multi-species primer DIA-16F (GGATCTCTTGGTTCTGGCAT) and reverse primer DIA-89R (ATGCCCACTAGAATACTAAT) were designed to anneal to the 5.8S rRNA gene region for all nine target Diatrypaceae species. Preliminary tests using conventional PCR showed the optimum annealing temperature for these primers was 58°C which amplified a single PCR product of 125 bp for all nine Diatrypaceae species tested (Figure 6.3; Table 6.1). No PCR products were observed with the DNA from nine Botryosphaeriaceae species and 19 other non-target species from grapevines (Table 6.1 and Table 6.2)

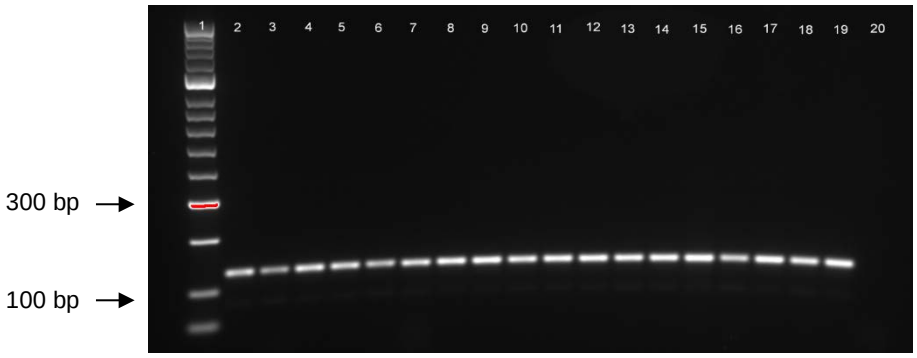


Figure 6.3. Representative 1% agarose gel of PCR products generated by multi-species primers 5S-16F – DIA-89F using genomic DNA of nine Diatrypaceae species. Lane 1, HyperLadder™ 50 bp (Bioline); lanes 2-6, *Eutypa lata*; lanes 7-9, *Cryptovalsa ampelina*, lane 10, *Eutypa leptoplaca*, lane 11-12, *Eutypella citricola*, lanes 13-14, *Diatrypella vulgaris*, lane 15, *Eutypella microtheca*, lane 16, *Eutypella cryptovalsoidea*; lane 17, *Eu. australiensis*, lanes 18-19, *Cryptovalsa rabenhorstii* and lane 20, non-template control. The numbers of the far left denote the molecular weights of the 50 bp ladder

6.3.1.5 BD qPCR using multi-species primers

For BD qPCR, Botryosphaeriaceae multi-species primer Bot BetaF1 (GTATGGCAATCTTCTGAACG) and Bot BetaR1 (CAGTTGTTACCGGCRCCAGA) which was degenerate at nucleotide 15 were further designed. These primers were expected to anneal to the β-tubulin gene region for all nine target Botryosphaeriaceae species. Preliminary tests using conventional PCR showed the optimum annealing temperature for these primers was 60°C which amplified a single 135 bp PCR product for all nine Botryosphaeriaceae species tested (Figure 6.4; Table 6.1). No PCR products were observed with the DNA of the nine Diatrypaceae species or 19 other non-target species from grapevines (Table 6.1). The standard PCR using serially diluted gDNA of *D. seriata* (A142) and *N. parvum* (B19a) demonstrated that the BotBetaF1 and BotBetaR1 multi-species primers could detect ~100 pg of gDNA while the nested-PCR protocol was able to detect as low as 10 pg of gDNA (data not shown).

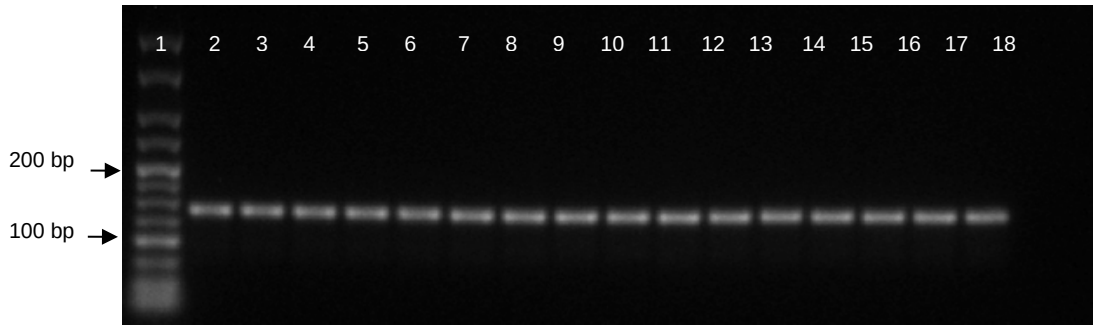


Figure 6.4. Representative 1% agarose gel of PCR products generated by multi-species primers BotBetaF1 and BotBetaR1 using genomic DNA of nine Botryosphaeriaceae species. Lane 1, HyperLadder™ 25 bp (Bioline); lanes 2-4, *Diplodia seriata*; lanes 5-7, *Neofusicoccum parvum*, lane 8-9, *Neofusicoccum luteum*, lane 10, *N. australe*; 11, *Botryosphaeria dothidea*; 12-13, *D. mutila*; lanes 14-15, *Lasiodiplodia theobromae*, lane 16*, Spencermartinsia viticola*, lane 17, *Dothiorella iberica*; lane 18, non-template control. The numbers of the far left denote the molecular weights of the 25 bp ladder.

6.3.1.6 Primer validation using international isolates

Validation of primers using the SuRDC isolate collection and equipment showed that all primers were able to amplify their corresponding target species while no PCR products were observed with any of the DNA from any non-target species further confirming the results of the specificity test conducted at NWGIC.

6.3.1.7 Efficiency of the DNA extraction protocols from spore tape samples

Of the four DNA extraction protocols tested, the protocol developed by Duvivier *et al*. (2009) and the Gentra PureGene kit gave consistent DNA yield while the other two protocols gave variable results (data not shown). Furthermore, the protocol by Rogers *et al.* (2009) also required reagents not readily available in Australia while the protocol by Duvivier *et al.* (2013) required hazardous chemicals including 2-mercaptoethanol and chloroform that can only be handled safely in a fume hood. However, the modified Gentra Puregene System was found to be easy to use and did not require toxic chemicals compared to the other protocols. DNA extracted from tape samples inoculated with 30 to 10,000 *N. luteum* conidia using the modified Gentra Puregene System were all amplified by BD qPCR using while tape samples inoculated with 100 to 10,000 *E. lata* ascospores were also amplified by ED qPCR (data not shown).

Table 6.1. Fungal isolates used for testing the specificity of Diatrypaceae multi-species primers (DITS1B-DITSR2 and 5S16F-DIA89R), Botryosphaeriaceae multi-species primers (Bot BetaF1-Bot BetaR1), Eutypa lata species-specific primers (BtEuF1-BtEuR1) and Cryptovalsa ampelina species-specific primers (CampBtF1 and CampBtR1) by conventional PCR. Isolates were obtained from the culture collections of Charles Sturt University (CSU), Agricultural Scientific Collections Unit (NSW DPI, Orange, NSW), Australia and Summerland Research and Development Centre (Summerland, BC, Canada).

Species	Origin	No. of isolates	Amplification for each primer pair[a]				
			DITS-1B & DITS-R2	5S-16F & DIA-89R	Bot BetaF1 & Bot BetaR1	BtEuF1 & BtEuR1	CampBtF1 & CampBtR2
Diatrypaceae species							
Cryptovalsa ampelina	*Australia*	8	-	+	-	-	+
	Canada, USA	3	-	+	-	-	+
Cryptovalsa rabenhorstii	*Australia*	2	+	+	-	-	-
Diatrypella vulgaris	*Australia*	2	+	+	-	-	-
Eutypa lata	*Australia*	11	+	+	-	+	-
	Canada, USA	2	+	+	-	+	-
Eutypa leptoplaca	*Australia*	6	+	+	-	-	-
Eutypella australiensis	*Australia*	1	-	+	-	-	-
Eutypella citricola	*Australia*	8	+	+	-	-	-
Eutypella cryptovalsoidea	*Australia*	1	+	+	-	-	-
Eutypella microtheca	*Australia*	4	+	+	-	-	-
Botryosphaeriaceae species							
Botryosphaeria dothidea	*Australia*	4	-	-	+	-	-
	Canada	2	-	-	+	-	-
Diplodia mutila	*Australia*	5	-	-	+	-	-
	Canada	2	-	-	+	-	-
Diplodia seriata	*Australia*	15	-	-	+	-	-
	Canada	2	-	-	+	-	-
Dothiorella iberica	*Australia*	4	-	-	+	-	-
Lasiodiplodia. theobromae	*Australia*	3	-	-	+	-	-
Neofusicoccum australe	*Australia*	4	-	-	+	-	-
Neofusicoccum parvum	*Australia*	7	-	-	+	-	-
	Canada	1	-	-	+	-	-
	**Ex-type specimen*	1	-	-	+	-	-
Neofusioccum luteum	*Australia*	5	-	-	+	-	-
	**Ex-type specimen*	1	-	-	+	-	-
Spencermartinsia viticola	*Australia*	4	-	-	+	-	-
	**Ex-type specimen*	1	-	-	+	-	-

[a] (+) = positive amplification; (-) = no amplification

*Ex-type specimens were purchased by SuRDC, Canada from CBS-KNAW Culture Collection, The Netherlands

Table 6.2. Fungal isolates of non-target species used for testing the specificity of Diatrypaceae multi-species primers (DITS1B-DITSR2 and 5S16F-DIA89R), Botryosphaeriaceae multi-species primers (Bot BetaF1 - Bot BetaR1), Eutypa lata species-specific primers (BtEuF1 - BtEuR2) and Cryptovalsa ampelina species-specific primers (CampBtF1 - Camp-BtR1). Isolates were obtained from the culture collections of Charles Sturt University (CSU), Agricultural Scientific Collections Unit (NSW DPI, Orange, NSW), Australia and Summerland Research and Development Centre, (Summerland, BC, Canada).

Species	Origin	No. of isolates	Amplification for each primer pair[a]				
			DITS-1B & DITS-R2	5S-16F & DIA-89R	Bot BetaF1 & Bot BetaR1	BtEuF1 & BtEuR1	CampBtF1 & CampBtR1
Other grapevine pathogens							
Aspergillus niger	Australia	1	-	-	-	-	-
Aspergillus sp.	Australia	1	-	-	-	-	-
Botrytis cinerea	Australia	3	-	-	-	-	-
Cadophora luteo-olivacea	Canada	3	-	-	-	-	-
Colletotrichum acutatum	Australia	3	-	-	-	-	-
Colletotrichum gloeosporoides	Australia	1	-	-	-	-	-
Greneria uvicola	Australia	2	-	-	-	-	-
Ilyonectria liriodendri	Australia	2	-	-	-	-	-
	Canada	3	-	-	-	-	-
Ilyonectria macrodidyma	Australia	4	-	-	-	-	-
	Canada	2	-	-	-	-	-
Penicillium sp.		1	-	-	-	-	-
Phaeoacremonium aleophilum	Australia	1	-	-	-	-	-
	Canada	2	-	-	-	-	-
	*Ex-type isolate	1	-	-	-	-	-
Phaeomoniella chlamydospora	Canada	3	-	-	-	-	-
	*Ex-type isolate	1	-	-	-	-	-
Phomopsis viticola	Australia	4	-	-	-	-	-
	Canada	3	-	-	-	-	-
Erysiphe necator	Australia	3	-	-	-	-	-
Other fungal species from grapevines							
Alternaria sp.	Australia	1	-	-	-	-	-
Chaetomium sp.	Australia	1	-	-	-	-	-
Cladosporium sp.	Australia	1	-	-	-	-	-
Conithyrium sp.	Australia	1	-	-	-	-	-
Epicoccum spp.	Australia	4	-	-	-	-	-
Fusarium spp.	Australia	2	-	-	-	-	-
Sordaria spp.	Australia	3	-	-	-	-	-

[a] (+) = positive amplification; (-) = no amplification

*Ex-type specimens were purchased by SuRDC, Canada from CBS-KNAW Culture Collection, The Netherlands

6.3.2 Development and optimisation of quantitative real time PCR (qPCR)

6.3.2.1 Development of gBlocks® Gene Fragments for qPCR standards

The 460 bp Diat-5S gBlock designed for ED qPCR was based on the internal fragment of rRNA gene, and the annealing sites for Dia16F and Dia89R primers are on the 233rd and 358th bp of the gene fragment. The 410 bp Bot-Btub gBlock that was designed for BD qPCR was based on the internal fragment of the β-tubulin gene, and the annealing sites for Bot BetaF1-Bot BetaR1 primers are on the 213th and 364th bp of the fragment. The mass of the Diat-5S gBlock was calculated as 0.50 attogram (ag)/copy (0.50e-18/copy) while the mass of the Bot-Btub gBlock was calculated as 0.45 ag/copy (0.45e-18g/copy). These are equivalent to 2.2×10^9 and 2×10^9 copies/ng for the Diat-5S and Bot-Btub gBlocks, respectively.

6.3.2.2 Development of ED qPCR using SYBR green

The Diat-5S gBlock and the gDNA of ED pathogens were successfully quantified by ED qPCR using SYBR green chemistry. A reaction efficiency of 98% with coefficients of determination of R^2 =0.98 was obtained based on the slopes of standard curves of the Diat-5S gBlock (Figure 6.5a). The analysis of the melt curve showed that the ED qPCR multi-species primers produced unique amplicons for both the gBlock and gDNA that resulted in a single peak at 83.5°C (±0.3) while no peaks were observed with NTC (H_2O) and non-target DNA (*N. luteum*; Figure 6.5b). This indicated that primers were specific only to the ED pathogen DNA and did not amplify non-specific targets.

The limit of detection (LOD) for the ED qPCR was 20 copies and 20 fg, using the Diat-5S gBlock standard and gDNA, respectively. This is equivalent to less than one spore. The mean Cq value for the sample containing 2 pg of *C. ampelina* (19.19) was not significantly different (*P*=0.212) from the Cq values of the same samples spiked with 200 pg of either *S. viticola* (19.14) or *L. theobromae* (19.10) indicating the ED qPCR method was not affected by the presence of non-target DNA.

The calculated number of copies of rRNA gene per nanogram of gDNA of the four representative ED pathogens by ED qPCR are presented in Table 6.3. Since the size of the *E. lata* haploid genome (or one spore) is 54 mbp or ~54 fg (Morales-Cruz *et al*. 2015), therefore, 1 ng of gDNA is equivalent to ~18,500 spores. Using these estimates, the average number of copies of rRNA gene in a haploid genome for the four representative Diatrypaceae species ranged from 105-114 (Table 6.3) and the mean value of 110 was used to estimate the number of spores in tape samples from vineyards.

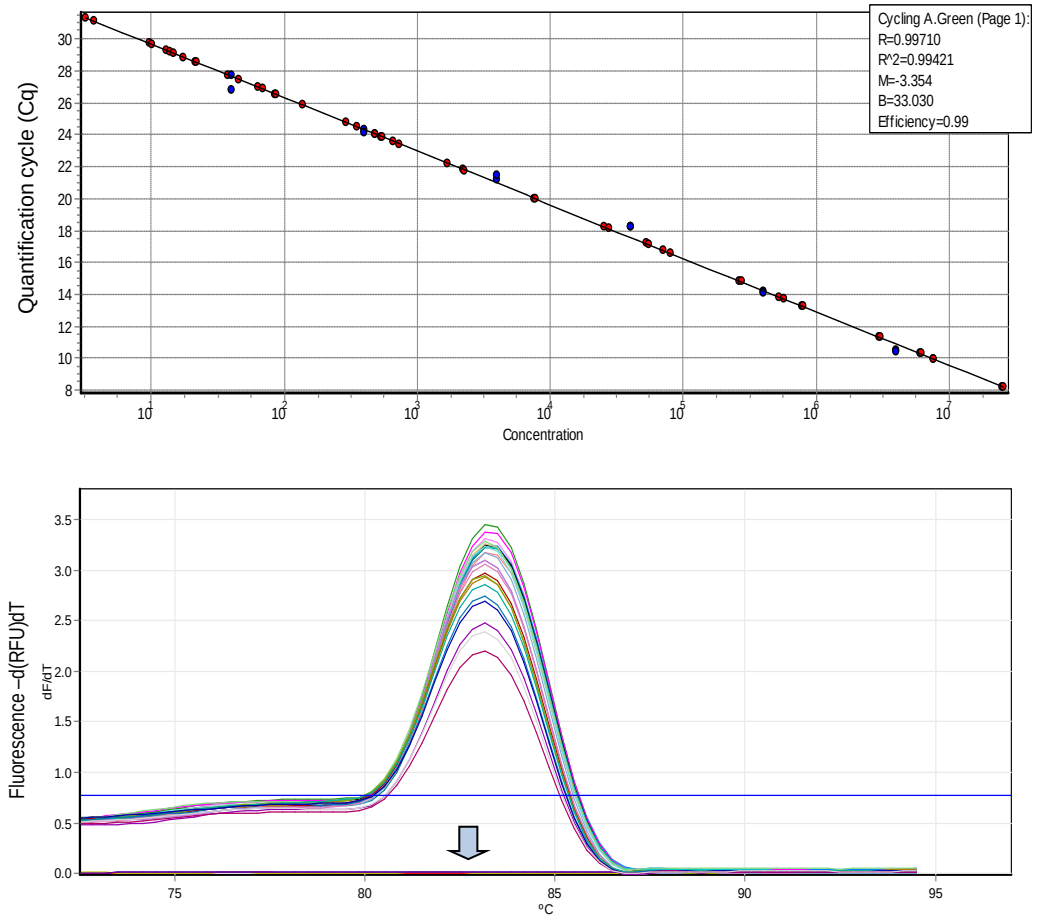


Figure 6.5. a) Standard curve of ED qPCR primers Dia-16F and Dia-89R using the Diat-5S gBlock gene fragments (blue dots) with a known number of copies as templates and with 10-fold serially diluted gDNA (red dots) from four representative Diatrypaceae species. The qPCR efficiency was 99% and the linear regression of standard curves gave a coefficient of determination of R^2=0.98; b) Melt curves obtained from the Dia-16F and Dia-89R products showing a single peak at 83.5 °C (±0.3) indicating a unique PCR product in both Diat-5S gBlocks and gDNA while no amplifications (arrow) were observed in both the non-template control (H2O) and negative control (*N. luteum* DNA).

Table 6.3. The calculated number of copies of the rRNA gene per haploid genome of the four Diatrypaceae species using Diat-5S gBlock gene fragments with known number of copies as standards. The resulting regression equations were used to quantify the number of copies of the rRNA gene per ng of gDNA.

Species	Mean no. of rRNA gene copies /ng of gDNA	*No. of rRNA gene copies in a haploid genome
Crytovalsa ampelina (CSU01)	1,942,500	105
Eutypa lata (ADSC-1)	2,039,750	110
Eutypa leptoplaca (RGA02)	2,119,115	114
Eutypella citricola (T3R2S2)	2,046,464	111
Mean	2,036,957	110

A. The calculated number of rRNA gene copies was based on the means of four technical replicates in two independent assays.
B. The number of rRNA gene copies was calculated based on the estimated size of *Eutypa lata* haploid genome of 54 mbp (~54 fg; Morales-Cruz *et al*. 2015).

6.3.2.3 Development of BD qPCR using TaqMan probe

The Bot-Btub gBlock and the gDNA of BD pathogens were successfully quantified by qPCR using TaqMan chemistry. A reaction efficiency of 94% with coefficients of determination of R^2=0.99 was obtained based on the slopes of the standard curve for the Bot-Btub gBlock (Figure 6.6). No amplifications were observed on NTC (H_2O) and non-target DNA (*E. lata*) indicating that the primers were specific only to the BD pathogen DNA and did not amplify non-specific targets.

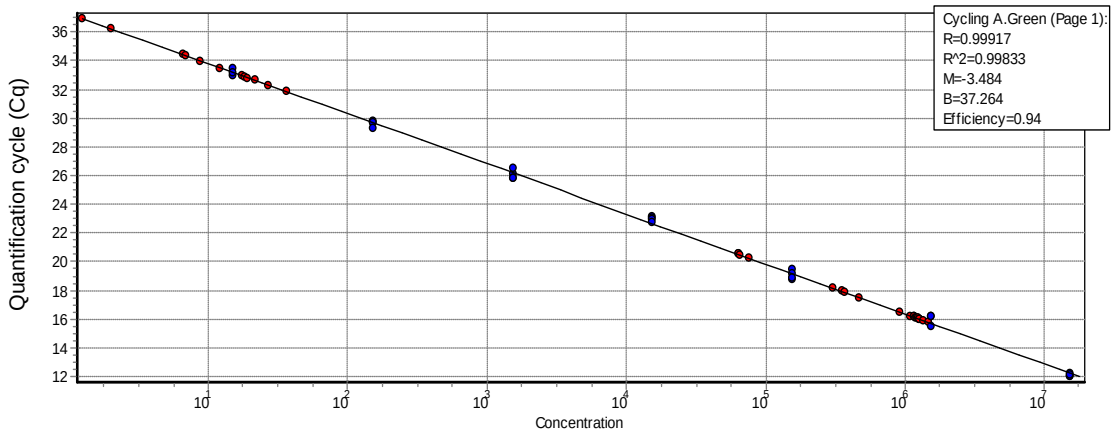


Figure 6.6. Standard curve of the BD qPCR primers Bot BetaF1 and Bot BetaR1 using the Bot-Btub gBlock gene fragments (blue) with a known number of copies as templates and with 10-fold serially diluted gDNA (red) from four representative BD pathogen species. The qPCR efficiency was 94% and the linear regression of standard curves gave a coefficient of determination of R^2=0.99.

For the BD qPCR, the limit of detection (LOD) was seven copies and 300 fg, using the Bot-Btub gBlock standard and gDNA, respectively. The calculated number of copies of β-tubulin gene per ng of Bot-Btub gBlock and in a haploid genome of the two BD pathogens are presented in Table 6.4. The calculated number of β-tubulin gene for *D. seriata* (A142) and *N. parvum* (B19) gene copies were 1.2 and 1.36, respectively (Table 6.4) indicating that the β-tubulin gene for BD species is a single copy gene as reported by Zhao, *et al*. 2014. The mean Cq value for the sample containing 2 pg of *D. seriata* (17.30) was not significantly different (*P*>0.05) from the same DNA sample spiked with 200 pg of either *C. ampelina* (17.29) and *E. lata* (17.32) indicating the BD qPCR method was not affected by the presence of non-target DNA (data not shown).

Table 6.4. The calculated number of copies of the β-tubulin gene per haploid genome of the two Botryosphaeriaceae species using Bot-Btub gBlock gene fragments with known number of copies as standards. The resulting regression equations were used to quantify the number of copies of the β-tubulin gene per ng of gDNA.

Species	[A]Mean no. of copies /ng of gDNA	[B]No. of β- tubulin gene copies in a haploid genome
Diplodia seriata (A142)	32,211	1.20
N. parvum (B19a)	32,488	1.36

A. The calculated number of β-tubulin gene copies per nanogram of genomic DNA (gDNA) was based on the means of four technical replicates in two independent assays.
B. The number of β-tubulin gene copies was calculated based on the estimated size of *Diplodia seriata* (37.1 mbp) and *Neofusicoccum parvum* (42.6 mbp) haploid genomes (Morales-Cruz, *et al*. 2015).

6.3.3 Spore trapping in vineyards

A total of 728 spore tape samples collected over four seasons for each site were analysed by nested PCR and 230 (31%) tested positive to either or both ED and BD pathogens, which were further analysed by qPCR. Preliminary results from qPCR showed spores of ED and BD pathogens were released sporadically at different times of the year in all four regions. Occasionally, both BD and ED spores were trapped in the same period and released continuously for several days. The spore release usually occurred during or after the occurrence of 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 any rainfall event. There was no obvious correlation between temperatures and spore release of both pathogen groups, with spores detected during summer and winter periods in all regions.

From the Barossa Valley analyses of the 179 spore tape samples collected from September 2013 to August 2014, 10 (6%) and 23 (13%) of the samples tested positive to ED and BD spores, respectively (Figure 6.7). For ED, spore release was similar throughout summer, autumn and winter (30% for each season) but less in spring (10%). The number of ED spores per sample ranged from 30-960 with the greatest number trapped in August 2013. For BD, spore release was greater in winter (35%) and spring (30%), compared to autumn (22%) and summer (9%). The number of BD spores per sample ranged from 8-300 spores with the greatest number trapped in November 2013. In winter 2013, 192 mm of rain fell over 53 days, 124 mm (16 days) in summer, 103 mm (25 days) in autumn and 84 mm (27 days) in spring. The high rainfall in summer was due to 96 mm of rain falling over a two-day period in February 2014, which coincided with release of BD spores only. However, on two occasions, ED and BD spores were also trapped seven days after a rain event in December.

From the Coonawarra analyses of 172 spore tape samples collected from December 2014 to mid-November 2015, 28 (16%) and 29 (17%) of the samples tested positive to ED and BD spores, respectively (Figure 6.8). For ED, spore release was greater in winter (54%) compared to autumn (32%), summer (11%) and spring (3%). The number of ED spores per sample ranged from 30 to 5,400 with the greatest numbers trapped in May (~2000), July (~1740) and September 2015 (~5,400). ED spores were detected continuously for six days on two occasions in winter. For BD, spore release was greater in winter (48%) compared to spring (21%), summer (17%) and autumn (14%). The BD spore numbers per sample ranged from 8 - 235 spores with the highest number trapped in September 2015. BD spores were detected continuously for six days on one occasion in winter and on three occasions was also detected 1-2 weeks after a rain event November. In winter, 133 mm of rain fell over 61 days which was greater compared with 107 mm (47 days) in autumn, 95 mm (20 days) in summer and 27 mm (11 days) in spring. For the spring season, spore trap data was only available up to mid-November 2015 due to a spore trap malfunction.

From the Griffith analyses of 182 spore tape samples collected from June 2014 to May 2015, 30 (17%) and 35 (19%) of the samples tested positive to ED and BD pathogens, respectively (Figure 6.9). For ED, spore release was similar across four seasons (20-30%) with spore numbers ranging from 30 to 2730 and the greatest numbers trapped in January 2015. ED spores were detected continuously for six days on one occasion in September 2014. However, on two occasions, ED spores were also detected one week after a rain event in August and April. For BD, spore release was greater in summer (54%) compared to spring (34%), winter (14%) and autumn (8%). The number of BD spores per sample ranged from 8 - 430 with the greatest number was also trapped in January 2015. BD spores were detected continuously for six and eight days on two occasions in September 2014 but on six occasions were also detected 1-2 weeks after a rain event across four seasons. In summer, 116 mm of rain fell in 21 days which was greater compared to 66 mm, (26 day) 60 mm (17 days), and 53 mm (16 days) for winter, spring and autumn, respectively. No rain was recorded in late July to mid-August and the entire month of March.

From the Hunter Valley analyses of 195 samples collected from June 2014 to May 2015, 41 (21%) and 35 (18%) of the samples tested positive to ED and BD pathogens (Figure 6.10). For ED, spore release was similar in spring, summer and autumn (27% for each season) but less in winter (19%). The number of ED spores was extremely high (up to 7500) with 10 samples from different seasons having more than 1000 spores and the greatest number trapped in May but on one occasion, ED spores were detected one week after a rain event in September. For BD, spore release was greater in autumn (34%) compared to winter (28%), summer (23%) and spring (14%). The number of BD spores per sample ranged from 8 - 227 with the greatest number trapped in May but on two occasions BD

spores were detected one week after a rain event in August 2014 and May 2015. In autumn, 404 mm of rain fell in 35 days (211 mm in two days for May). This amount of rain is much greater compared to 298 mm (44 days) in summer, 114 mm (25 days) in winter and 86.4 mm (19 days) in spring. To investigate the high prevalence of ED spores detected and despite *E. lata* not being reported in the region (Creaser *et al*. 2003; Trouillas *et al*. 2011), four representative samples that tested positive using the ED multi-species primers in conventional PCR were sent for DNA sequencing at the Australian Genome Research Facility (agrf.com.au). The BLAST analyses of the sequences identified these samples as *Eu. microtheca* and not *E. lata* (data not shown).

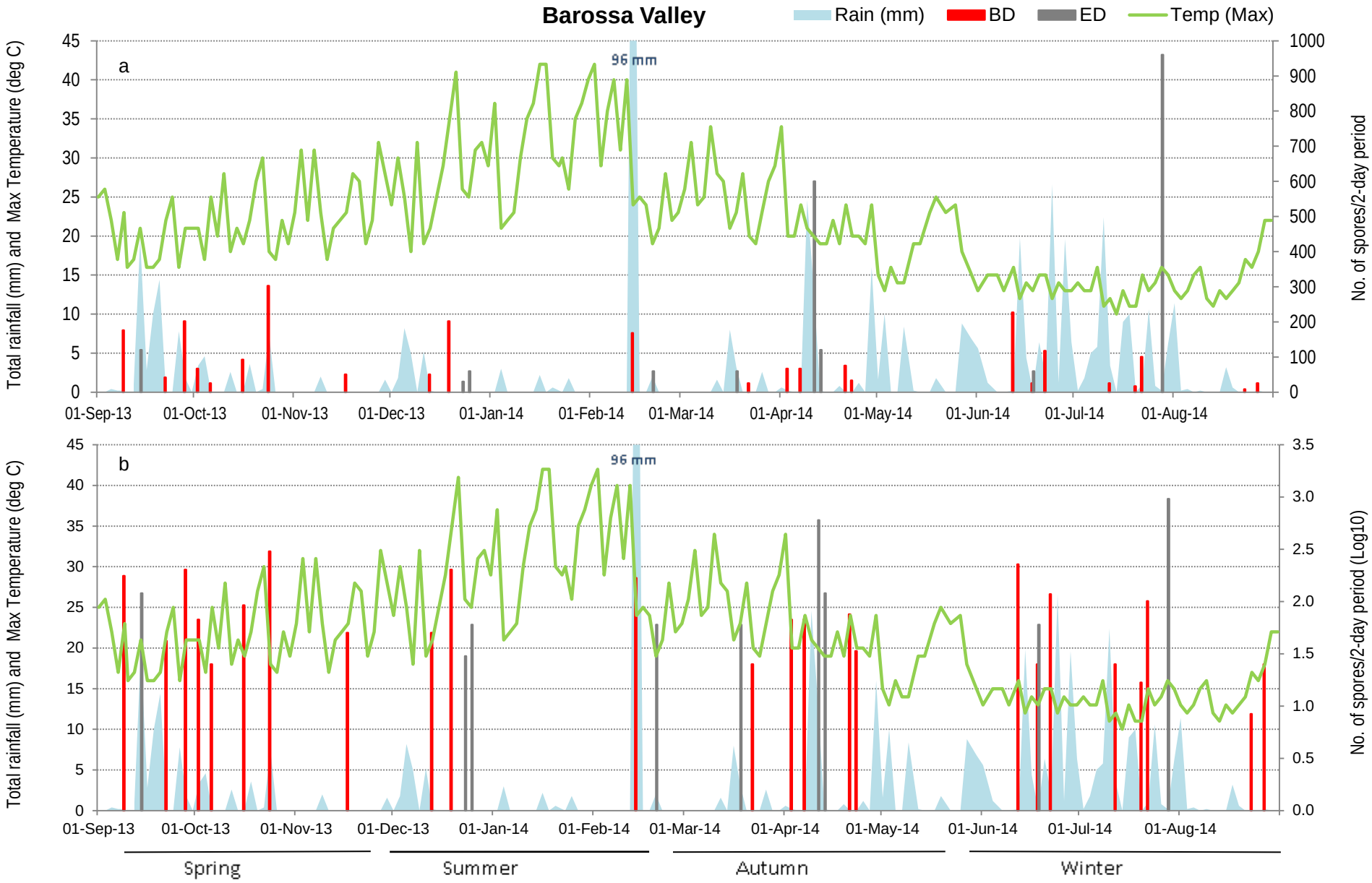


Figure 6.7. Number of spores (a) and Log_{10} transformed (b) of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogens trapped per 2-day period in the Barossa Valley, South Australia, from September 2013 to August 2014, plotted against rainfall and maximum temperature (www.bom.gov.au).

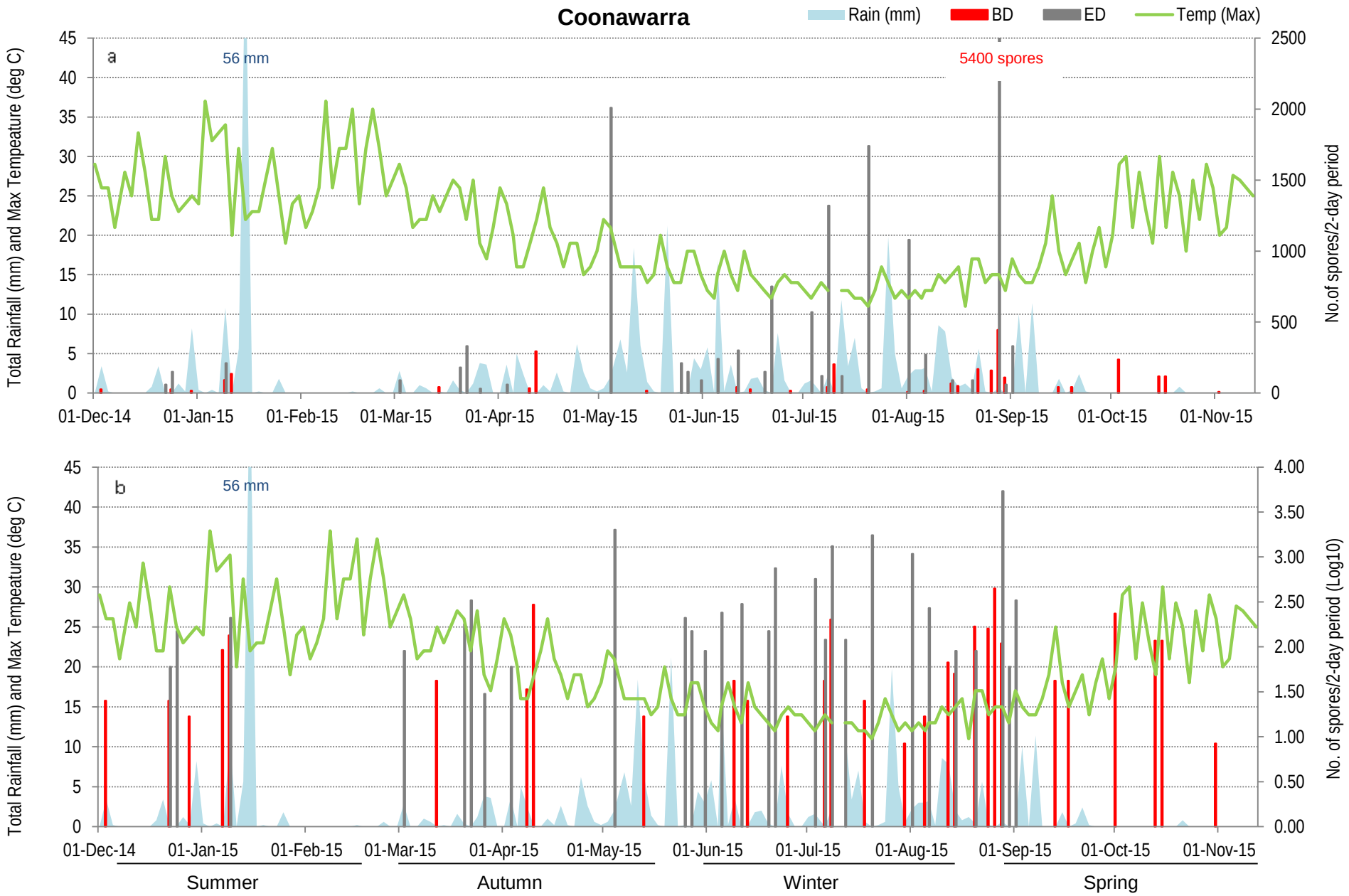


Figure 6.8. Number of spores (a) and Log_{10} transformed (b) of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogens trapped per 2-day period in Coonawarra, South Australia, from December 2014 to mid-November 2015 and plotted against rainfall and maximum temperature (www.bom.gov.au).

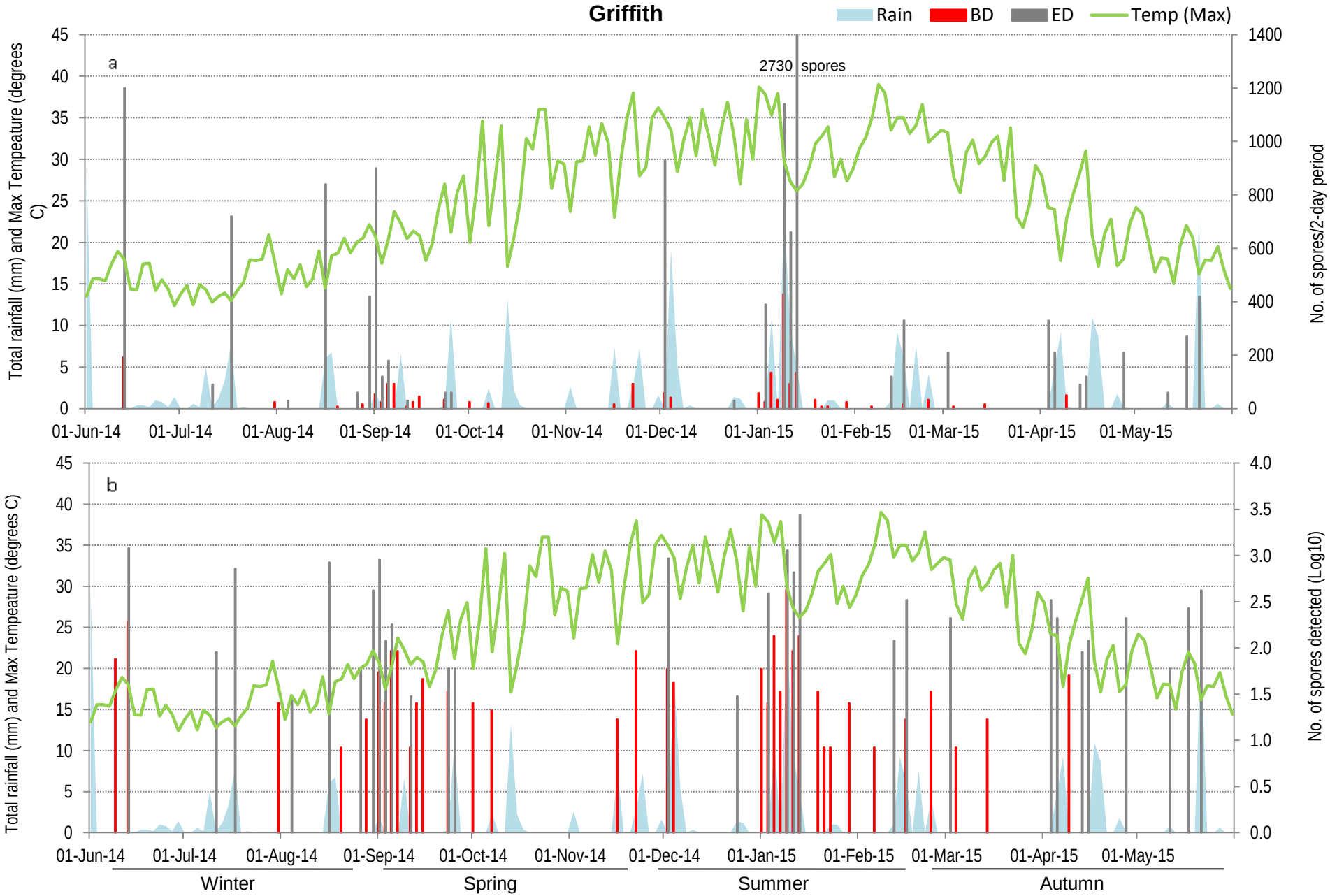


Figure 6.9. Number of spores (a) and Log_{10} transformed (b) of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogens trapped per 2-day period in Griffith, New South Wales, from June 2014 to May 2015 and plotted against rainfall and maximum temperature (www.bom.gov.au).

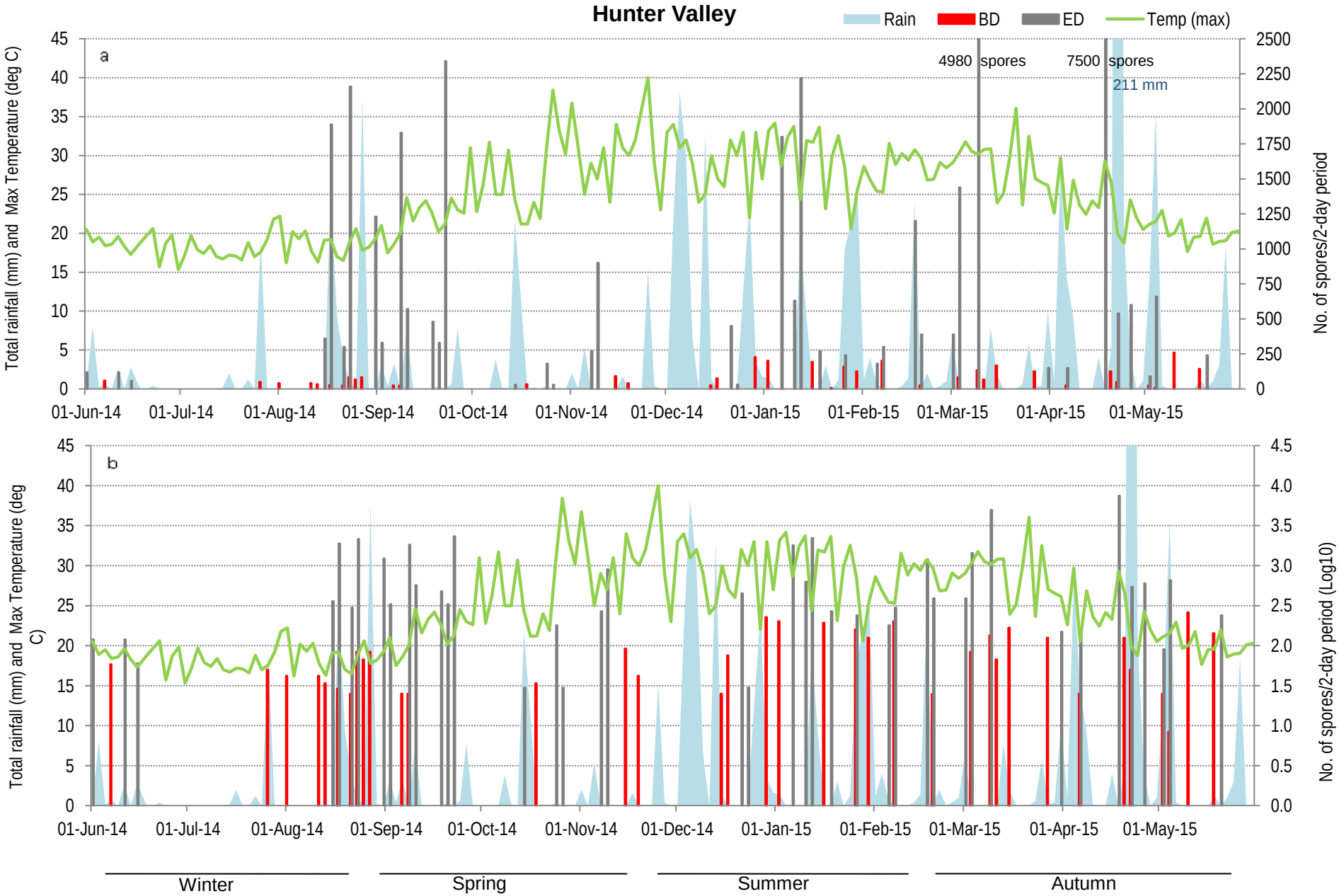


Figure 6.10. Number of spores (a) and Log_{10} transformed (b) of Eutypa dieback (ED) and Botryosphaeria dieback (BD) pathogens trapped per 2-day period in the Hunter Valley, New South Wales, from May 2014 to September 2015 and plotted against rainfall and maximum temperature (www.bom.gov.au).

6.4 Discussion

6.4.1 Development of molecular tools to detect and quantify ED and BD spores

The initial objective of this study was to develop highly specific and sensitive molecular tools for the detection and quantification of spores for several species of ED and BD pathogens in vineyards. Previous studies on spore dispersal patterns of ED (Moller and Carter 1965; Pearson 1980; Petzoldt *et al*. 1983; Ramos *et al*. 1975; Trese *et al*. 1980; Trouillas 2009; van Niekerk *et al.* 2010), and BD pathogens (Amponsah *et al.* 2009; Urbez-Torres *et al.* 2009; Valencia *et al*. 2015; van Niekerk *et al.* 2010) relied primarily on conventional techniques like microscopy and plating that are extremely time consuming, less accurate and less sensitive than molecular tools in detecting these pathogens from environmental samples. In recent years, the use of DNA-based molecular tools has become popular for the detection and quantification of different plant pathogens. Quantitative PCR has been used to quantify airborne inoculum of *Sclerotinia sclerotiorum* (Rogers *et al*. 2009), *Leptosphaeria maculans* in canola (Van de Wouw, *et al.* 2010), and *Mycosphaerella graminicola* in wheat (Duvivier, *et al*. 2013). In grapevines, qPCR assays were also used to quantify spores of *Botrytis cinerea* (Diguta *et al.* 2010) and *Phaeomoniella chlamydospora* and *Phaeacremonium aleophilum* (Martin *et al.* 2012). This method was further used to quantify *Sphaeropsis sapinea* infections from *Pinus nigra shoots.* However, none of these primers were designed to detect multiple species of pathogens in a single sample. Spagnolo *et al.* (2010) and Ridgway et al. (2011) developed multi-species primers that can detect low amounts of DNA of multiple Botryosphaeriaceae species but these primers were designed primarily for conventional PCR that is only semi-quantitative. Recently, multiplex qPCR has become popular in quantifying multiple target DNA in a single sample. This technology works by using two to five species-specific primers and their specific probes that are labelled with different fluorophores and combined in a single reaction tube. Pouzoulet *et al*. (2013) successfully quantified two pathogens (*Ph. chlamydospora* and *P. aleophilum)* from infected wood samples by multiplex qPCR. However, the primers and probes for multiplex PCR are more difficult to design and optimise, as well as more costly when compared with singleplex qPCR (Bio-rad Laboratories, 2006). The qPCR protocols using multi-species primers developed in this study are easier and more cost effective options than multiplex-PCR but can accurately detect and quantify up to nine target species in a single sample. To our knowledge, these are the first qPCR methods that have been developed to target multiple species of ED and BD pathogens from environmental samples (in this case from spore trap samples).

For spore trapping, Melinex tapes coated with petroleum-based adhesive was used to allow the spores to stick to the tape surface. The removal of spores from the sticky surface, however, was difficult and affected the DNA yield during extractions. The tapes contain adhesives, other microorganisms, insects and dust that become trapped on the surface and that can be inhibitory to qPCR. Thus, a DNA extraction protocol that can efficiently remove spores from the tapes without compromising the DNA quantity and quality was essential for this study. Rogers *et al.* (2009) and Duvivier *et al.* (2013) developed DNA extraction protocols from spore tapes but these methods require reagents that are either not available in Australia or are hazardous to humans, and require special equipment during handling. In this study, we modified the protocol from the Gentra® Puregene® (Qiagen) system that allowed the extraction of DNA from spore tapes by using lysing tubes that contain two types of ceramic beads. The beads allowed the rapid scraping of the spores from the tape surfaces as well as breaking the spore cell walls so that DNA can be released and isolated. This method was shown to give consistent yields of DNA when used for extracting DNA from artificially-inoculated tapes. This method was also found to be safe and easy to use and was therefore used as the standard protocol for extracting DNA from all vineyard spore trap tapes.

Two sets of Diatrypaceae multi-species primers DITS1B–DITSR2 and 5S16F-DIA89F were developed for conventional and qPCR, respectively, targeting nine Diatrypaceae species associated with ED in Australian vineyards. A multi-species primer and Taqman probe (Bot-BetaF1 - BotBetaR1) for BD qPCR were also developed to detect and quantify nine Botryosphaeriaceae species commonly associated with BD in vineyards. Furthermore, two sets of species-specific primers targeting the most prevalent Diatrypaceae species in Australia, namely *E. lata* (BtEuf1 and BtEuR1) and *C. ampelina* (CampBtF1 and CampBtR1) were further developed. Specificity tests conducted at the NWGIC laboratories demonstrated that all primers were able to amplify the DNA of their corresponding target species while no PCR products were observed with the DNA of all the other non-target species tested, including other grapevine fungal pathogens. These results were further validated at SuRDC in

Canada using their isolate collection from different countries and the validation results confirmed all primers were highly specific to their target species.

The two qPCR protocols developed for this study were able to distinguish between the target species (in this case nine Diatrypaceae or Botryosphaeriaceae species) and other fungal DNA. The quantification of the target DNA for each protocol was not affected by the presence of non-target DNA in the qPCR reactions. The qPCR protocol for ED pathogens can detect up to 20 copies of the rRNA gene or 20 fg of gDNA which is equivalent to less than one spore. The qPCR protocol for BD pathogens, on the other hand, can detect up to seven copies of the β-tubulin gene or 300 fg of gDNA and is equivalent to seven spores. The limit of detection for ED qPCR was 20 fg which is more sensitive than the qPCR developed for *Ph. chlamydospora* by Martin *et al*. (2013) with a detection limit of 108 fg of gDNA using the multi-copy rRNA gene as target. The sensitivity of BD qPCR is similar to the qPCR methods for *S. sclerotiorum* by Rogers *et al.* (2009) and *P. aleophilum* by Pouzoulet *et al.* (2013) with a detection limit of 350 fg and 250 fg of gDNA, respectively, for their respective single-copy target gene.

The sensitivity of the two qPCR protocols was affected by the copy number of the target DNA sequence in the spore. In this case, the target rRNA gene sequence of the ED qPCR primers (Diat16F and Diat89R) occurs in multiple copies (50 to 110 in different fungal haploid genome), thereby increasing its detection sensitivity. However, the number of rRNA repeats for *E. lata* and other Diatrypaceae species is currently unknown, therefore, calculating the number of spores based on the rRNA copies is difficult. The BD qPCR primers (BotBetaF1 and BotBetaR1) on the other hand target the β-tubulin gene which occurs in single copies in a genome (Zhao, *et al*. 2014) making it less sensitive but easier to translate into spore numbers (in this case one copy is one spore). The BD qPCR also requires greater amounts of DNA template (7 µL) compared to ED qPCR that only requires 2 µL of template. Many qPCR studies involve cloning the genes of interest into a bacterial plasmid in order to generate single copies of the target genes, and use these cloned genes as standards (Duvivier *et al.* 2013; Pouzoulet *et al.* 2013). Instead of cloning, which is difficult and time consuming, this study used gBlocks® which are synthetic single copies of the two target genes (rRNA and β-tubulin) and used as the qPCR standards. These gBlocks® are commercially synthesised by Integrated DNA technologies and therefore are cost effective and easy to prepare. This allowed the determination of the number of rRNA genes by plotting different concentrations of gDNA of four representative Diatrypaceae species with the gBlocks® standards. Using the size of the *E. lata* haploid genome (~54 mbp or ~54 fg DNA mass; Morales-Cruz *et al*. 2015) as a reference, we were able to estimate the copy number of rRNA genes in a haploid Diatrypaceae genome to ~110 copies.

The two multi-species primers developed for ED and BD qPCR in this research are rapid and inexpensive tools to detect and quantify multiple ED and BD pathogens in a single qPCR run. However, due to their multiple targets, these primers cannot discriminate to species level within a sample. Identification of the different species from individual spore trap tapes will determine the most prevalent species that contribute to the spore release in each region. Studies conducted by Ridgway *et al*. (2010) combined PCR with single-stranded conformation polymorphism (SSCP) analysis to resolve the identities of the mixed Botryosphaeriaceae species amplified by their multi-species primers. A DNA-based molecular diagnostic tool (DNA macroarray) was shown to detect several species of pathogens causing young vine decline from a single nursery planting material, including Petri/Esca disease and Black foot (Urbez-Torres *et al*. 2013). The macroarray for ED fungi is currently being developed by Dr. Úrbez Torres and his team at SuRDC while the macroarray for BD is yet to be developed. The PCR-SSCP system or the DNA macroarray can potentially be used in the future for identification of different ED or BD species present in spore tape samples. However, both DNA macroarray and SSCP require special equipment (i.e. hybridisation oven and chemiluminescent imaging system for DNA macroarray) that is currently unavailable at the NWGIC.

6.4.2 Spore trapping in Australian vineyards

This study was the first attempt to comprehensively investigate the spore release patterns of both ED and BD pathogens in four wine regions of Australia using molecular tools. A previous study on BD spore release in the Hunter Valley (2009-2011), using glass slides and rainwater traps, was found to be time consuming and only yielded semi-quantitative data (Savocchia, unpublished; NWG 06/01). Information on spore dispersal of ED fungi in Australia was also based on a study in apricot orchards conducted in the 1960s (Moller and Carter 1965). Preliminary data analysed to date from the Barossa Valley, Coonawarra, Hunter Valley and Griffith showed that BD and ED spores were released at

different times of the year, but the seasonal release and number of these spores differed between pathogens and regions. The current data from this study showing rainfall as a primary factor that triggers the release of either ED or BD spores are in general agreement with past studies in vineyards in California (Petzoldt *et al*. 1983; Ramos *et al*. 1975; Trouillas 2009; Urbez-Torres, *et al*. 2009), 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).

The studies from overseas revealed that the seasonal release of these spores varied depending on the climate of each region, which is similar to the results we obtained from four different regions in Australia. In the Barossa Valley, more BD spores were trapped than ED spores while in Coonawarra, the spore numbers were similar between ED and BD pathogens. In contrast, the number of ED spores trapped in Griffith and the Hunter Valley were slightly higher than BD. The results obtained particularly from the Barossa Valley and Hunter Valley were unexpected since *E. lata* is believed to be more prevalent in cooler regions such as the Barossa Valley (Highet and Wicks, 1998), while 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 spores trapped in the Hunter Valley and Griffith may be attributed to other Diatrypaceae spores since the four representative samples from Hunter Valley were identified as *Eu. microtheca* and not *E. lata*. The survey by Trouillas *et al.* (2011) showed that *Eu. microtheca* was the most prevalent species isolated from the Hunter Valley while *E. lata* was not isolated from any grapevines in this region. The factors that affect the number of ED spores in the Barossa Valley are still unclear and require further investigation. Analyses of the remaining samples from these regions may further confirm these results, but more intensive sampling within regions will be necessary to determine the variation in spore release by the different pathogens across a region. Identification of the ED and BD species that were trapped in each region using either PCR-SSCP or DNA macroarray described in Section 6.5.1 may also assist in elucidating the prevalent species involved in the spore release.

Differences in seasonal release of ED and BD spores between regions may also be attributed to different climatic conditions in each region. In the Barossa Valley and Coonawarra, a higher percentage of ED and BD spores were detected in winter and less in summer which is opposite to the results obtained from Griffith and the Hunter Valley where more spores were detected during summer. Although the amount of rain that fell during the winter season in all four regions was similar, the rainfall in the Barossa Valley (2014) and Coonawarra (2015) was more widespread with a total of 53 and 61 days of rain recorded during each season, respectively. The rainfall in winter 2014 and 2015 for the Hunter Valley and Griffith on the other hand, was less frequent with only 25 days and 32 days of rain recorded, respectively. However, more widespread rain was recorded in summer 2015 in the Hunter Valley (44 days) while only 16 days of rain were recorded in the Barossa Valley in summer 2014. The rain events in Coonawarra and Griffith during these seasons were similar and so spore number differences between the two regions may be attributed to the location of the spore trap in Griffith, which was close to a citrus orchard, similar to that of the closest vines (20 m). The Diatrypaceae and Botryosphaeriaceae species were reported as causing citrus branch canker (Mayorquin *et al*. 2016). In California, the most prevalent Diatrypaceae species isolated from citrus were *Eu. citricola* and *Eu. microtheca* while the most prevalent Botryosphaeriaceae species was *B. dothidea* (Mayorquin *et al.* 2016). Since the citrus orchard at Griffith may also harbour the ED and BD pathogens, this could be an alternative source of inoculum that may have contributed to the high number of spores trapped at this site. Since only one year of spore trapping data has been analysed to date, the analyses of the remaining samples by July 2017 will further clarify the seasonality of the spore release between regions. Furthermore, the weather data used for the analyses of spore release patterns was only limited to rainfall and maximum temperature. Thus, there is a need to investigate the effects of other environmental factors, including relatively humidity, dew point, minimum temperatures and wind speed, in spore release for these pathogens. Consultation with a climatologist may further assist in incorporating other environmental data that will further elucidate the spore release patterns of these pathogens.

The data generated from this study clearly showed that spore release of ED and BD pathogens was associated with rain events, which is in agreement with other studies conducted elsewhere (Pearson, 1980; Trese *et al*. 1980; Kunztmann *et al*. 2009; Urbez-Torres, *et al*. 2009; van Niekerk, *et al*. 2010). At all four sites, ED and BD were generally trapped 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 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. The fruiting bodies of the ED and BD pathogens have long life spans. According to Moller and Carter (1965), the stroma of *E. lata* is productive for more than one year and new perithecia develop when the old ones are exhausted. Dubos (1987; cited by Carter 1991) reported that once exhausted, fruiting bodies required around 12 days for maturation of new ascospores. Amponsah *et al*. (2008) also showed that Botryosphaeriaceae species in dried wood pieces can still produce pycnidia and conidia after three months in storage at room temperature. However, these dried wood pieces require 36 hours of humidity before they produce new pycnidia and conidia. The amount of time for the pathogens to develop new fruiting structures and produce spores may explain the lack of spores trapped in some rainy periods. Further work is required, however, to determine the duration of the production and maturation of perithecia and/or pycnidia of these pathogens. Furthermore, on few occasions at different locations, spores were trapped more than a week after any rainfall event. The spore release during these dry periods may be attributed to other environmental factors (e.g. relative humidity, dew, wind speed) but will require further investigation.

Another important observation obtained from this study was the difference in the amount of spores released by ED and BD pathogens, with larger numbers of ED spores detected (up to 7500 spores) in a 2-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 away) 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*. 2009). Since the BD pathogens are less airborne, the distance of their dispersal in vineyards is most likely shorter than for ED spores (Urbez-Torres *et al*. 2009). 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 look at airborne inoculum. 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 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.

The studies from overseas described previously also revealed the seasonal release of ED and BD spores to vary depending on the region. In California, the greatest number of BD spores was trapped primarily in autumn and winter but 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 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 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. There is no information on the risk of infection during spring and summer pruning in Australia, and this would need to be the focus of future research.

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. Trials on pruning wound susceptibility to infections by *E. lata* (McLaren Vale) and *D. seriata* and *N. luteum* (Wagga Wagga) showed that wounds were most susceptible during the first two weeks following pruning (Section 7). While wounds seem 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, there may be little advantage in using different pruning times to minimise the risk of infections of these pathogens. However, the pruning wound protection trials (Section 8) 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 four major wine regions in this study provided beneficial information on the spore release patterns of ED and BD pathogens in different regions. The molecular tools developed in this study fast-tracked the analyses of the spore trap samples and allowed quantification of ED and BD spores from vineyards. The data generated from samples analysed to date demonstrated that the seasonal spore release patterns of ED and BD pathogens varied between pathogens and regions. It also identified rainfall as the primary environmental factor that influenced the release of these pathogen spores. Once the data are generated from all three years of spore trapping at each location, this study will elucidate the critical times of the year when ED and BD spores are abundant in vineyards. In current research (SAR 1601), six spore traps have been deployed across Australian wine regions and will be monitored for a further three years to expand our knowledge on the breadth of climatic differences that exists between wine regions of Australia. 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.

7 PRUNING WOUND SUSCEPTIBILITY

7.1 Introduction

The susceptibility of grapevine pruning wounds to pathogens causing eutypa and botryosphaeria dieback (ED and BD) has been the subject of many studies worldwide. In Australia, the only research to date was conducted on apricots almost 50 years ago (Carter and Moller 1967, 1970) and indicated that wounds were susceptible to infection by *E. lata* for up to four weeks during early winter, with decreased susceptibility in late winter and early spring. Since then, research on grapes in temperate climates of California (Moller and Kasimatis 1980, Petzoldt *et al* 1981 and Munkvold and Marois 1995) and south-west France (Chapuis *et al*. 1998) indicated high wound susceptibility for periods up to seven weeks in early winter down to low susceptibility for periods of only two weeks in late winter and early spring. However, in Michigan (Trese *et al* 1982) and South Africa (van Niekerk *et al*. 2011) the trend was for higher susceptibility in late winter and early spring than in early winter. For species that cause botryosphaeria dieback, wounds were susceptible for up to 16 weeks in Italy (Serra et al. 2008), up to 12 weeks in California (Úrbez-Torres and Gubler 2011) and up to 21 days in South Africa (van Niekerk *et al.* 2011). Furthermore, the degree of susceptibility throughout the pruning season varied greatly between these countries. Therefore it is imperative that the susceptibility of grapevine pruning wounds to infection by grapevine trunk disease pathogens be evaluated in Australian environments.

7.2 Effect of pruning time

7.2.1 Methods

Trials were established in 2013, 2014 and 2015 on Shiraz vines planted in 1996 (McLaren Vale, SA) and Cabernet Sauvignon vines planted *circa* 1990 (Wagga Wagga, NSW).

In McLaren Vale, one-year-old canes were pruned to two buds on 4 June, 16 July and 27 August 2013 using secateurs (Figure 7.1a). For each pruning time, inoculation time treatments of 1, 7, 14, 28, 42 and 56 days after pruning were applied. A non-inoculated control (NIC) was also included at each pruning time. At each inoculation time, wounds were moistened by spraying with sterile distilled water (SDW) immediately prior to inoculation with approximately 500 spores of *E. lata* suspended in 20 µL SDW and applied using a pipette (Figure 7.1b). Each treatment was allocated to a vine with 10 pruned canes. The trial was set up as a randomised block design with 10 replications. The trial was repeated in 2014 with pruning occurring on 3 June, 15 July and 26 August and again in 2015 with pruning on 2 June, 14 July and 25 July.

In Wagga Wagga, one-year-old canes were pruned to two buds on 4 June, 23 July and 20 August 2013 using secateurs. For each pruning time, inoculation time treatments of 1, 7, 14, 28, 42, 56, 84 and 112 days after pruning were applied. A non-inoculated control was also included at each pruning time. At each inoculation time, wounds were moistened by spraying with SDW immediately prior to inoculation with approximately 1000 spores of *Diplodia seriata* suspended in 20 µL SDW and applied using a pipette. Each treatment was allocated to a vine with 10 pruned canes. The trial was set up as a randomised block design with 10 replications. In 2014 the trial was repeated, with pruning occurring on 12 June, 22 July and 22 August and again in 2015 using *Neofusicoccum luteum* instead of *D. seriata* for inoculations on 19 June, 20 July and 17 August.

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 25,000 spores/mL using a haemocytometer.

Diplodia seriata was grown from storage on autoclaved ~10 cm grapevine canes (cv. Cabernet Sauvignon) in Petri dishes lined with moist filter paper and incubated at 25°C in 12:12 light:dark regime for 3-4 weeks. *N. luteum* was grown on PDA amended with chloramphenicol (100 mg/L) and incubated at 25°C in total darkness overnight before incubation under UV light (12:12 light:dark) for

4-5 weeks. For both species, spores were washed with SDW from the plates and/or canes and the concentration adjusted to 5 x 10^4 spores/mL using a haemocytometer.

E. lata 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, 12 hr day/ night light cycle. After 24hrs, using a compound microscope at 200x magnification, the number of non-germinated spores / 100 in each of the drops was counted and a mean percentage of germinated spores was determined.

D. seriata and *N. luteum* spore germination was assessed by plating 100 µL of the spore suspension onto PDA plates and spreading with a sterile culture spreader (hockey stick), at 3 – 4 replicates per plate. Plates were incubated at 25°C for 5-7 days and colony forming units (cfu) were counted. The mean number of CFUs per plate was divided by 100 (the expected CFUs per plate). The viability of the spores was never below 72% for any of the species used.

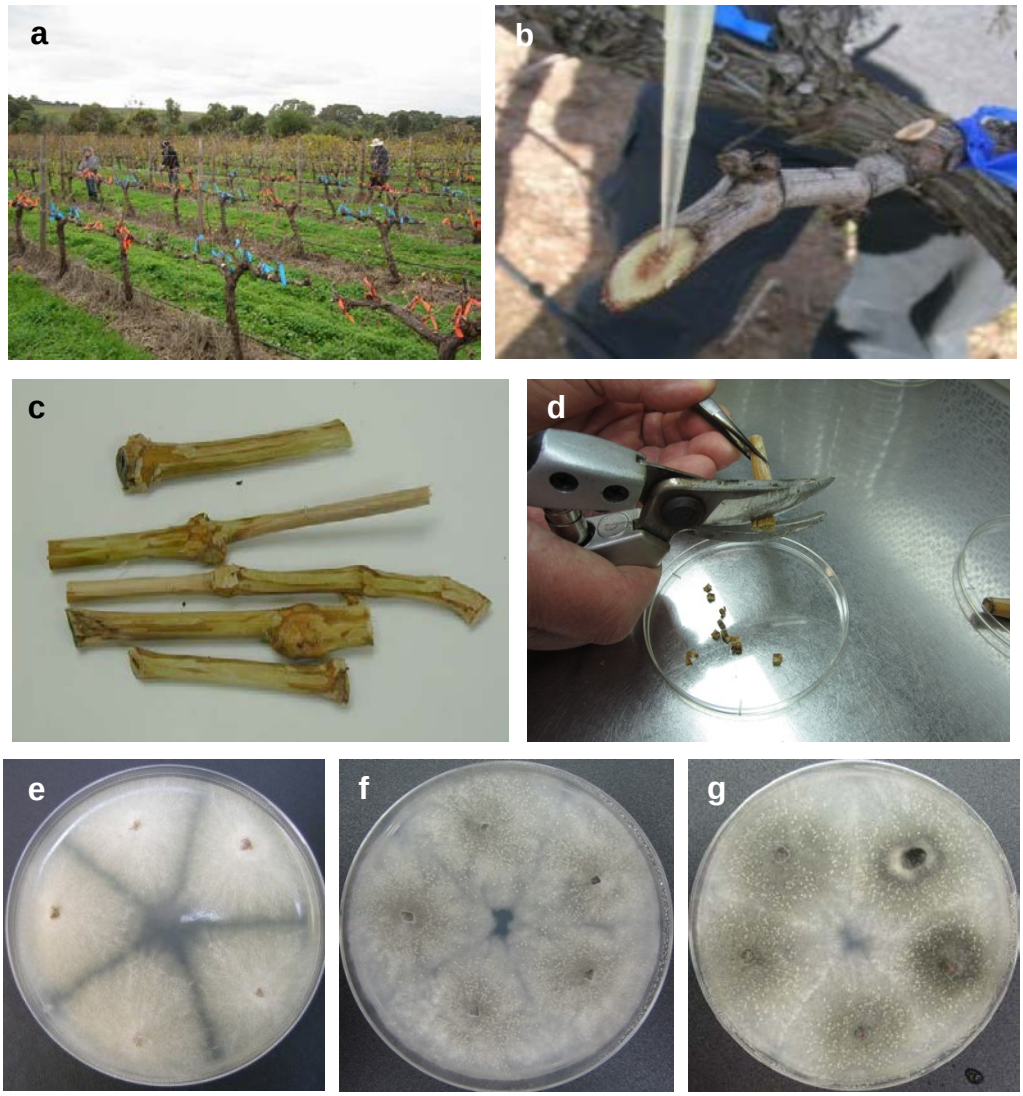


Figure 7.1. Methods used to evaluate susceptibility of wounds in the vineyard: (a) cutting canes to two-bud spurs on Shiraz vines at McLaren Vale; (b) inoculating wounds with ascospores of *Eutypa lata* (c) spurs with bark removed; (d) cutting chips from segments that spanned the interface between normal and stained wood and (e) cultures of *E. lata*, (f) *Diplodia seriata* and (g) *Neofusicoccum luteum* growing from wood chips.

For each trial, canes were harvested from vines between April and June, 12 months after 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 sterilised in 2.5% sodium

hypochlorite (NaOCl) for 10 minutes (*E. lata*) or 3 minutes (*D. seriata*/*N. luteum*) and then washed twice in SDW. Using flame-sterilised 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 each plastic Petri dish filled with PDA amended with antibiotic (streptomycin sulfate 100 mg/L), with two plates used for each cane for ED experiments. For BD experiments, five canes were randomly selected from each vine treatment; 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*, *D. seriata* or *N. luteum* (Figure 7.1e-f). Data were subjected to analysis of variance and least significant difference (LSD) at the 5% level was used for all pairwise comparisons; standard error of the means were calculated.

Climate data for McLaren Vale were provided by Measurement Engineering Australia (MEA) and the McLaren Vale Grape, Wine and Tourism Association, from an on-site automatic weather station (AWS) approximately 30 metres from the trial site. Climate data for Wagga Wagga were obtained from an AWS located 1.5 km from the trial site (www.bom.gov.au).

7.2.2 Results

7.2.2.1 Eutypa dieback - McLaren Vale, SA

In 2013-14 recovery from wounds inoculated with *E. lata* was significantly lower for the vines pruned mid-season (July, 53% recovery when inoculated one day post-pruning) than for early-pruned vines (June, 78%; Figure 7.2a). With the late pruning in August, recovery at one-day post-pruning was 65%, not significantly different from the other two pruning times. At all three pruning times, recovery of *E. lata* was reduced significantly to between 16-22% (early), 8-34% (mid) and 6-38% (late) when inoculated at 7 and 14 days post-pruning. Recovery rates for wounds inoculated 28 to 56 days post-pruning were similar to those for non-inoculated controls (0-8% for all three pruning times).

In 2014-15, although there was a reduction in recovery of *E. lata* from day 1 to day 14 from 88 to 29% and 59 to 22% for the early and late pruning times, respectively, there was low recovery of *E. lata* from the 1 to 14-day inoculations following the mid-pruning, with recovery ranging from 24 to 7%. Recovery rates from wounds inoculated 28 to 56 days post-pruning were similar to those for non-inoculated controls (0-10% for all three pruning times).

In 2015-16, susceptibility of wounds to infection was significantly lower for the vines pruned at mid and late times (76 and 73% recovery of *E. lata*, respectively when inoculated one day post-pruning) than for those pruned early (99%; Figure 7.2c). At day 7 for all three pruning times there was no significant difference in recovery (78, 66 and 71% for early, mid and late pruning times, respectively). For the early pruning time, recovery from the day 14 inoculation was 75%, similar to the day 7 inoculation, whereas for the mid and late pruning times, recovery was significantly reduced to 29 and 26%, respectively, at the 14 day inoculation. There was no difference in recovery between the early, mid and late pruning times when inoculated at day 28, although recovery was significantly reduced compared with the 14 day inoculation (27-31%). Recovery rates from wounds made 42 to 56 days post-pruning were similar to those for non-inoculated controls (3-28% for all three pruning times).

For each year, rainfall was scattered throughout June to September, corresponding to the lowest temperatures and solar radiation, and the highest humidity for the year (Figure 7.3). Temperature and solar radiation increased from mid-September each year, corresponding to a decrease in humidity. Mean total daily solar radiation was lowest at the early pruning time in June for each of the three years with 1.9, 2.0 and 2.2 kWh/m^2 recorded in 2013, 2014 and 2015, respectively, corresponding with the highest recovery of *E. lata* for each year, compared with 3.0, 3.4 and 3.0 kWh/m^2 for the late pruning times in August of each year. Maximum temperatures were 17, 16 and 25 °C, 24, 12 and 18°C and 15, 18 and 22 °C for the early, mid and late pruning times in 2013, 2014 and 2015, respectively.

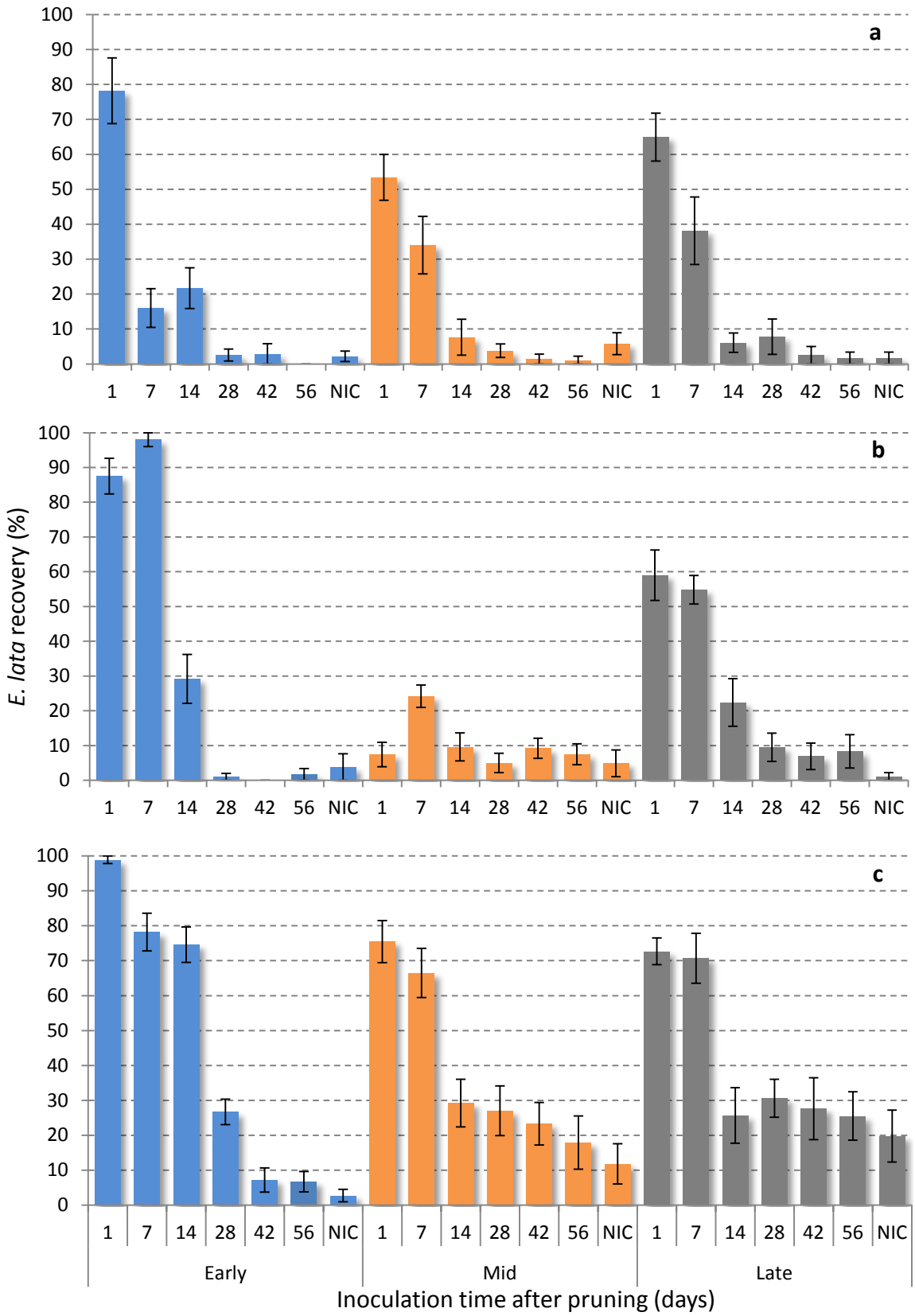


Figure 7.2 Incidence of recovery of *Eutypa lata* at the McLaren Vale site from canes pruned on 4, 3 and 2 June (Early), 16, 15 and 14 July (Mid) and 27, 26 and 25 August (Late) in 2013-14 (a), 2014-15 (b) and 2015-16 (c), respectively, and inoculated with 500 spores at 1, 7, 14, 28, 42 and 56 days after pruning. NIC = non-inoculated control. Bars represent standard error of the mean.

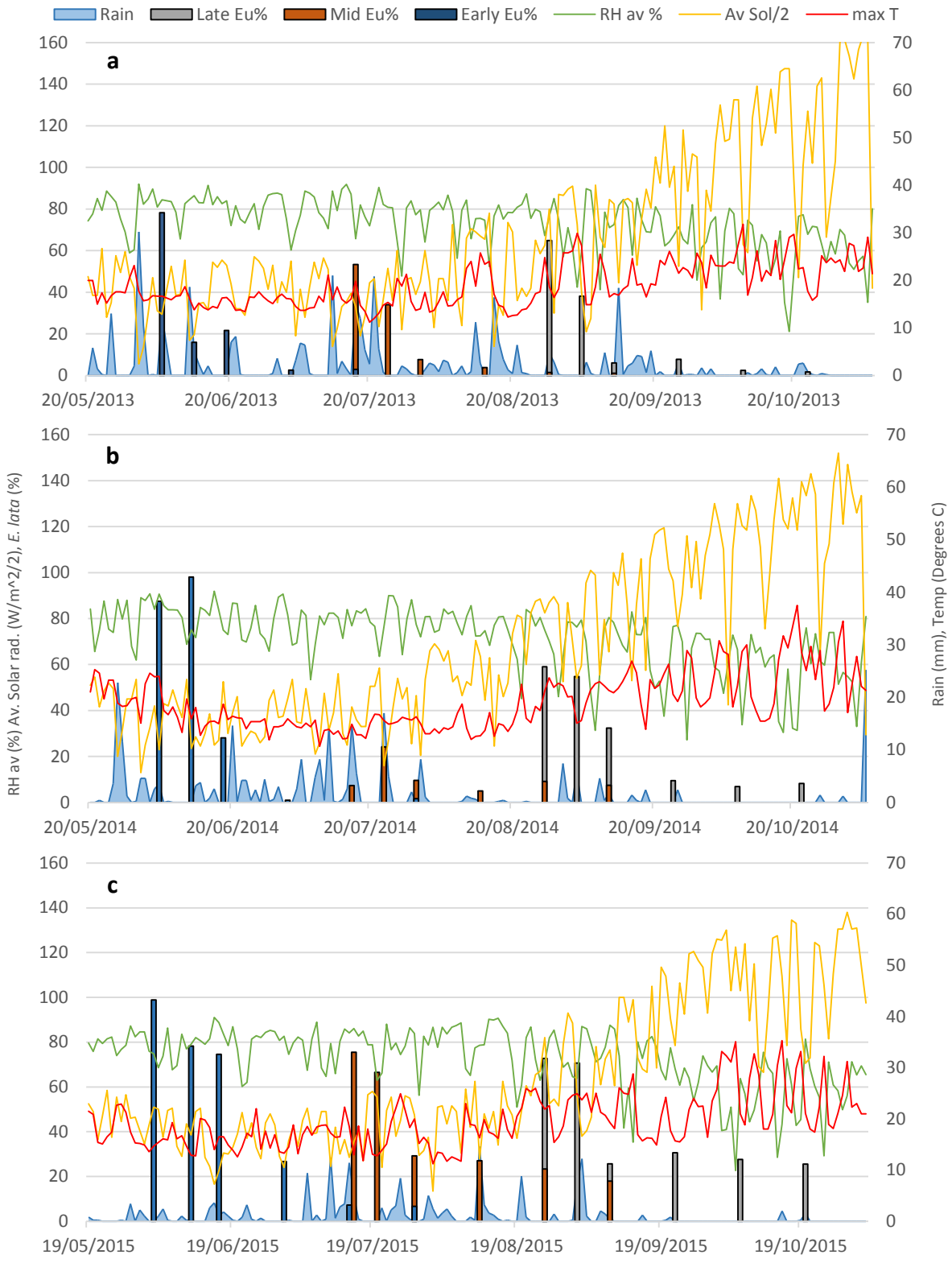


Figure 7.3 Weather data and *Eutypa lata* recovery percentage (solid bars) associated with the six inoculation times (1, 7, 14, 28, 42 and 56 days) following each pruning time (early-June, mid-July and late-August) at the McLaren Vale trial site for 2013-14 (a), 2014-15 (b) and 2015-16 (c). Values on the left axis are daily average relative humidity (%), daily average solar radiation (W/m^2/2) and recovery of *Eutypa lata* (%) and on the right axis are total daily rainfall (mm) and maximum daily temperature (°C).

7.2.2.2 Botryosphaeria dieback - Wagga Wagga, NSW

In 2013-14, susceptibility of wounds to infection by *D. seriata* was significantly lower for those vines pruned late (August) with 50% recovery when inoculated one day post-pruning than for those pruned early (June) and mid (July, 100%; Figure 7.4a). For early-pruned vines, *D. seriata* recovery was high at the 7 day inoculation, similar to that of the 1 day inoculation, but inoculations from 14-112 days yielded no more infection than that of the non-inoculated control. In mid-pruned vines, recovery from inoculations made at 7-28 days decreased significantly from 96 to 60%, and for inoculations made from 56 to 112 days, recovery was not different to the non-inoculated controls. In late-pruned vines, non-inoculated controls yielded significantly more *D. seriata* than wounds inoculated at any times.

In 2014-15, susceptibility of wounds to infection by *D. seriata* was similar, regardless of when the vines were pruned, with 94-100% pathogen recovery for all pruning times when inoculated 1 - 14 days post-pruning (Figure 7.4b). The incidence of recovery of *D. seriata* was significantly reduced for inoculations made 28–112 days post-pruning, with pathogen recovery incidence 42-66% from early-pruned vines and 22-88% in mid- and 50-72% in late-pruned vines. Natural infection by *D. seriata* was reflected in 22-36% pathogen recovery from non-inoculated controls for all three pruning times.

In 2015-16, susceptibility of wounds to infection by *N. luteum* for the day 1 inoculation was lowest for the late pruning, with 64% pathogen recovery, compared with early at 84% and mid at 98% (Figure 7.4c). For the early and mid-pruning times for the day 7 inoculations, recovery had decreased to 64 and 56%, respectively, while for the late pruning time, recovery was reduced to 20%. When inoculated at 14 and 28 days, recovery further reduced to 22 - 26% (early pruning), 10-14% (mid) and 2-14% (late). From 56-112 day inoculations, recovery was negligible and *N. luteum* was not recovered from non-inoculated controls.

For each year, rainfall was scattered throughout May to December, and generally corresponded to decreased temperature and solar radiation (Figure 7.5). Temperature and solar radiation increased from early August. Cumulative rainfall totals for the seven days preceding each of the pruning times were notably higher in 2013 than in 2014, with 58, 20 and 10 mm for the early, mid and late pruning times, respectively, in 2013, and 16, 0.2 and 0 mm in 2014. This higher rainfall corresponded to higher recovery of *D.* seriata from the non-inoculated controls in 2013 than 2014.

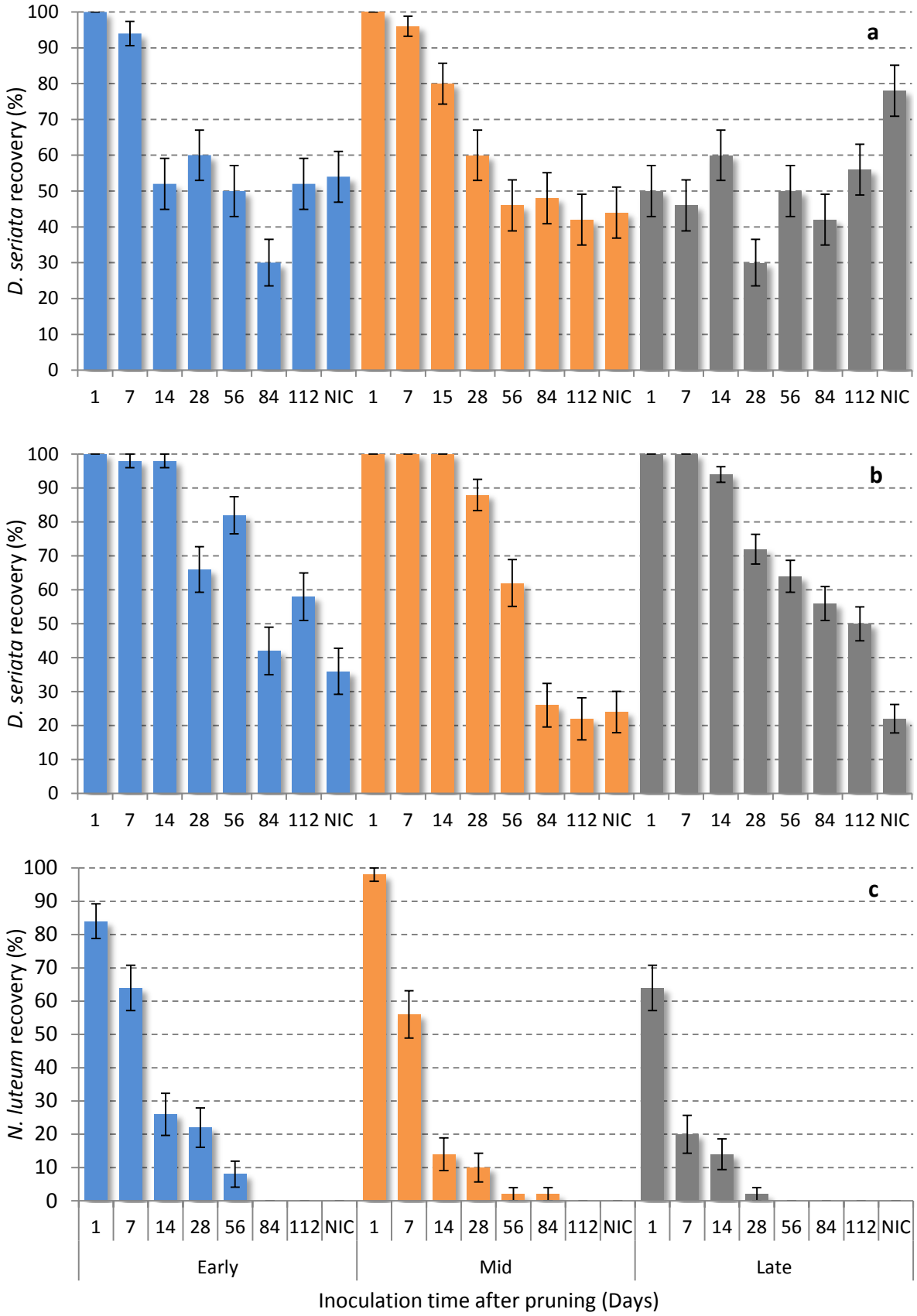


Figure 7.4 Incidence of recovery of *Diplodia seriata* (a and b) and *Neofusicoccum luteum* (c) from canes pruned on 4, 12 and 19 June (Early), 23, 22 and 20 July (Mid) and 20, 22 and 17 August (Late) 2013-14 (a) and 2014-15 (b) and 2015-16 (c), respectively, and inoculated with 1000 spores at 1, 7, 14, 28, 42, 56, 84 and 112 days after pruning. NIC = non-inoculated control. Bars represent standard error of the mean.

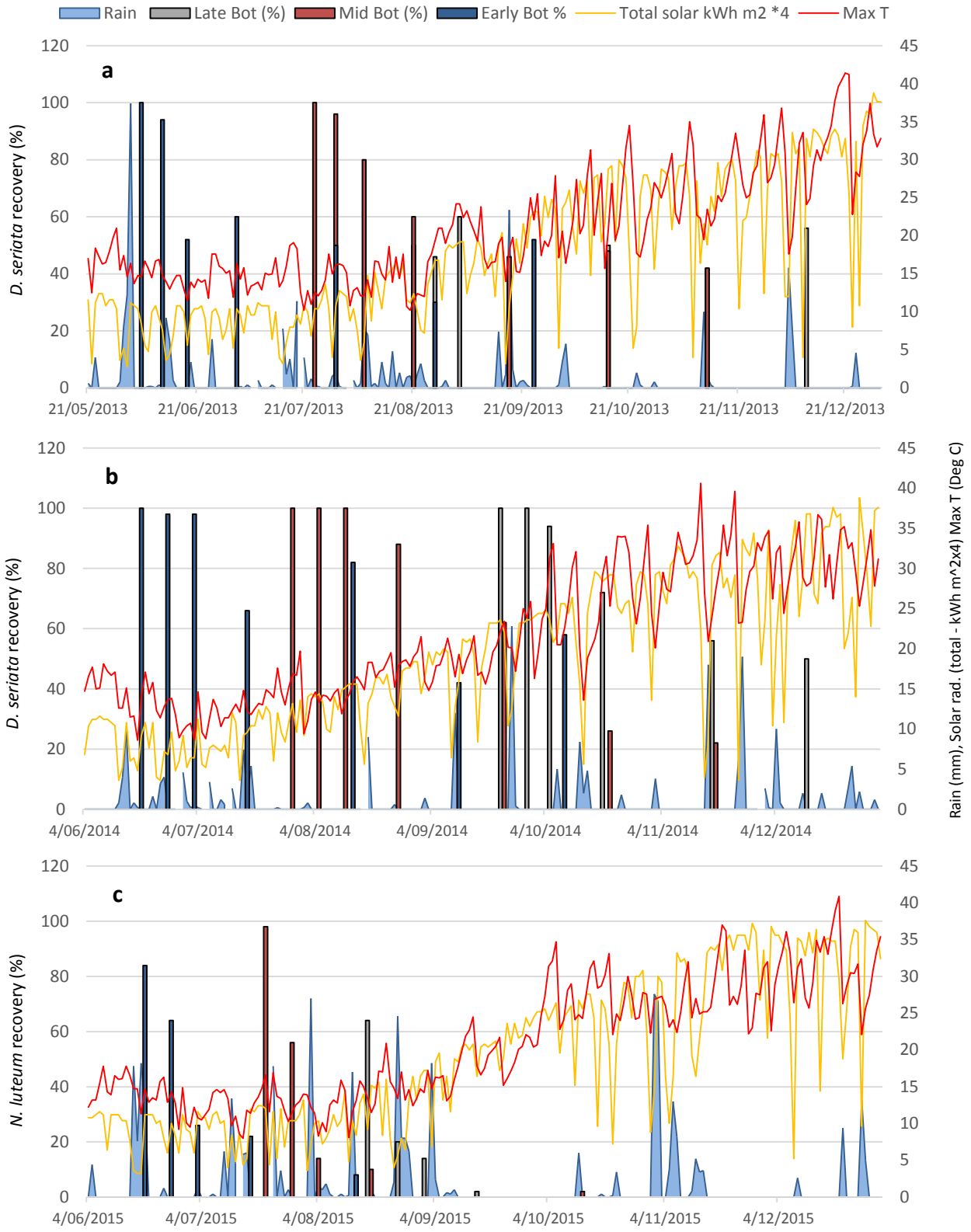


Figure 7.5 Weather data and *Diplodia seriata* or *Neofusicoccum luteum* recovery percentage (solid bars) associated with the seven inoculation times (1, 7, 14, 28, 56, 84 and 112 days) following each pruning time (early-June, mid-July and late-August) at the Wagga Wagga trial site for 2013-14 (a), 2014-15 (b) and 2015-16 (c). Values on the left axis are recovery of *Diplodia seriata* or *Neofusicoccum luteum* (%) and on the right axis are total daily rainfall (mm), total daily solar radiation (kWh/m^2 x4) and maximum daily temperature (°C)

7.3 Cultivar variation

7.3.1 Methods

For the ED and BD cultivar susceptibility experiments, grapevine canes (cvs. Cabernet Sauvignon, Chardonnay, Merlot, Sauvignon Blanc, Semillon and Shiraz) were collected in 2013 and 2014 from the University of Adelaide Waite Campus vineyard in Urrbrae, SA during dormancy and stored at 3-4ºC. These cultivars were chosen as they are the six most planted cultivars in Australia. A detached cane assay (Ayres *et al.* 2011) experiment was established on 20 February 2014 and repeated on 26 February 2015. Canes 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 approximately 1 cm below the boards (Figure 7.6a). Boards were set up to hold 10 treatments and eight canes per treatment were placed in rows across the boards. The experiments were set up as randomised block designs with five replications (boards). The boards with canes were floated on tap water in plastic tubs (Figure 7.6b) in a greenhouse maintained at approximately 25°C.

Wounds on top of canes were moistened by spraying with SDW immediately prior to inoculation with approximately 500 spores of *E. lata* (ED) or *N. luteum* (BD) suspended in 20 µl SDW and applied using a pipette (Figure 7.6b). Wounds were inoculated 1, 7 or 14 days after they were made. Two canes of each cultivar per replication remained non-inoculated as controls. The viability of the spores in the inoculum was assessed by culturing and was never below 77% at any of the inoculation times.



Figure 7.6 Detached cane assay; a) single node cutting, b) inoculating cuttings inserted into polystyrene boards floating on water, c) growth of *Eutypa lata* (left) and *Neofusicoccum luteum* (right) from wood segments.

Four weeks after the 14 day inoculation, canes were removed from boards and processed as described in Section 7.2.1, and assessed for presence or absence of *E. lata* or *N. luteum* (Figure

7.6c). Data were subjected to analysis of variance and least significant difference (LSD) at the 5% level was used for all pairwise comparisons; standard error of the means was calculated.

7.3.2 Results

In SA, there was no difference between results for the two eutypa dieback DCA experiments, so combined data are shown in Figure 7.7. There was no significant difference in recovery of *E. lata* between cultivars at the three inoculation times evaluated. However, for all six cultivars, recovery of *E. lata* from wounds inoculated seven days after they were made (62-73%) was significantly less frequent than from those inoculated after one day (83-98%). While recovery decreased again for wounds inoculated at 14 days (30-48%), the differences were not always statistically significant.

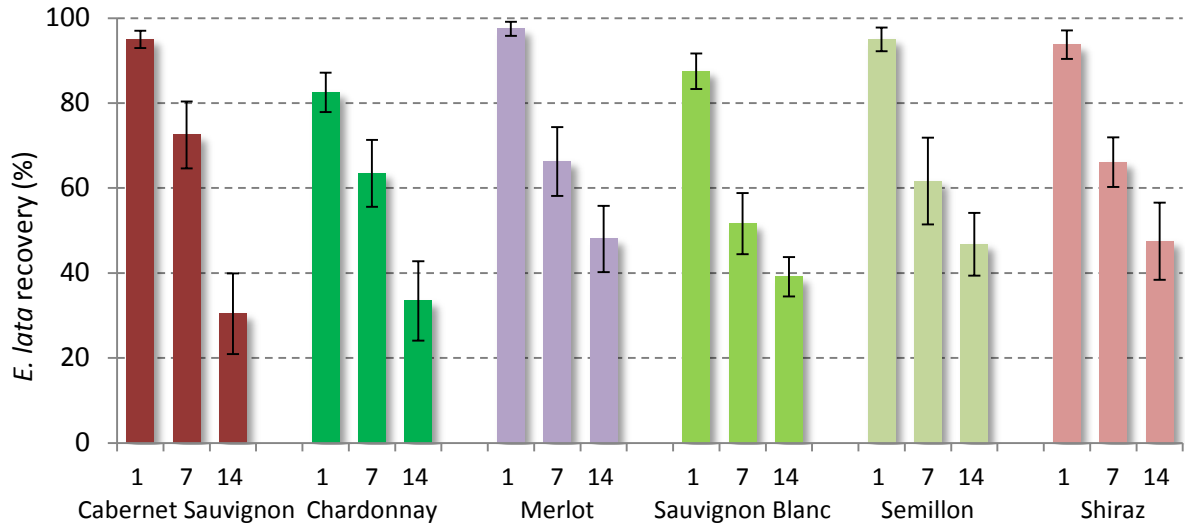


Figure 7.7 Incidence of *Eutypa lata* recovery from detached canes which were pruned and then inoculated with 500 spores at 1, 7 and 14 days after pruning. Data combined for repeated experiments. Bars represent standard error of the mean.

In NSW there was no difference in results for the two botryosphaeria dieback DCA experiments, so combined data are shown in Figure 7.8. There were no significant differences in recovery of *N. luteum* between cultivars for the day 1 inoculation. The only significant reduction in recovery from wounds inoculated at 7 days was for Semillon (78%, reduced from 99%), however, recovery from the day 14 inoculation was reduced from day 1 for all cultivars except Cabernet Sauvignon.

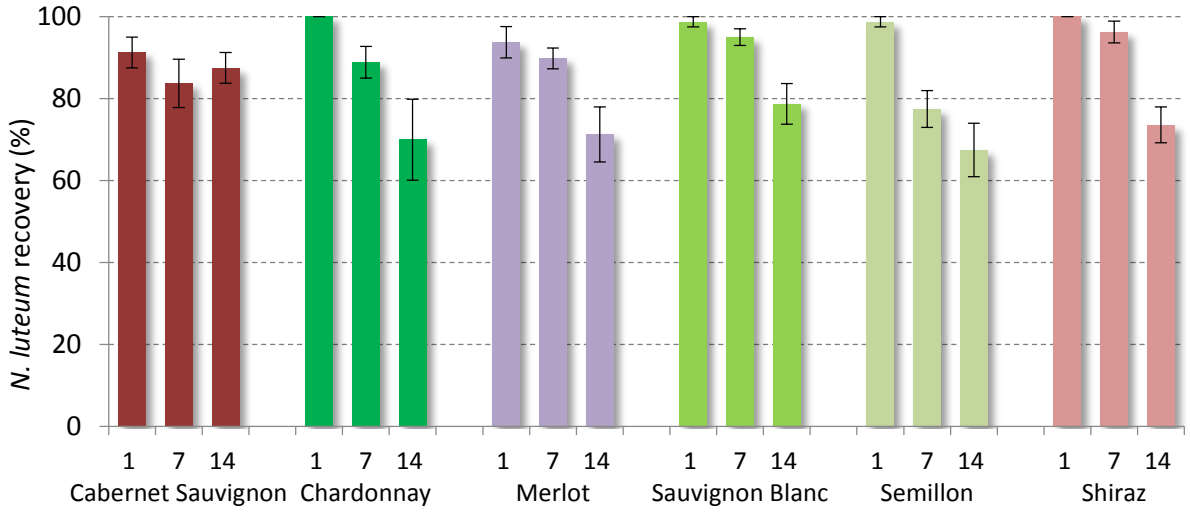


Figure 7.8 Incidence of *Neofusicoccum luteum* recovery from detached canes which were pruned and then inoculated with 500 spores at 1, 7 and 14 days after pruning. Data combined for repeated experiments. Bars represent standard error of the mean.

7.4 Discussion

Results of field trials 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. Susceptibility of pruning wounds to infection by *E. lata* (in McLaren Vale) and *N. luteum* (in Wagga Wagga) decreased more rapidly than did susceptibility to infection by *D. seriata* (in Wagga Wagga). In general, wounds were more susceptible to *E. lata* when pruned early (June) than at the later pruning times, susceptibility to *N. luteum* was least at the latest pruning time, and there was no difference in susceptibility to *D. seriata* between pruning times. This is the first extensive report on grapevine pruning wound susceptibility in Australia. Elsewhere, research has revealed that patterns of wound susceptibility varied, with pruning wounds in California and south-west France more susceptible to trunk disease pathogens in early winter than in late winter/early spring (Moller and Kasimatis 1980, Petzoldt *et al*. 1981, Munkvold and Marois 1995 and Chapuis *et al*. 1998), with the opposite reported in Michigan and South Africa (Trese *et al*. 1982 and van Niekerk *et al*. 2011a). These differences can be attributed to climatic differences between the regions, and this study has highlighted the importance of evaluating wound susceptibility in Australian regions in varying climates.

Trese *et al.* (1982) suggested that in Michigan, cold weather post-pruning (<5°C) led to reduced infection by *E. lata* whereas Chapuis *et al.* (1998) found, in trials conducted in France, that the mean percentage of wounds infected by *E. lata* decreased as temperature increased. van Niekerk *et al.* (2011a), in the Stellenbosch region of the Western Cape province of South Africa, found that late winter wounds were more susceptible to infection than wounds made earlier in the season, but temperature and rainfall varied little over the period of interest. In McLaren Vale, there was no significant difference in the mean monthly temperature for the early, mid and late pruning times for each year of the trial but analysis of the monthly mean daily solar radiation exposure showed that the lowest solar radiation occurred in June for each of the three years. It may be possible that this is linked to the higher recovery of *E. lata* from the early pruning time, however, this would need to be confirmed with further research.

In McLaren Vale during 2014-15, recovery from the mid pruning time wounds was unusually low for the inoculation one day after pruning, not different from the non-inoculated control. According to weather data collected at the site, there were no obvious climatic events that may have led to the low recovery. Furthermore, germination tests were undertaken prior each inoculation, and revealed high germination for the suspension prepared for the inoculation in question (data not shown). Therefore, the most likely reason for the poor recovery was experimental error, in that wounds for this particular treatment may have been inadvertently missed at inoculation time.

Natural infection by *E. lata*, as indicated by recovery from the non-inoculated controls, was relatively low each year, ranging from 1.1 to 5.8%, with the exception in 2015-16 of the mid and late-pruned vines, where it was 11.8 and 19.8%, respectively. This greater level of natural infection may explain the relatively high recoveries from the day 28 to 56 inoculations following those pruning times, as infection may have occurred soon after pruning. However, analysis of the weather data from those pruning times did not reveal any obvious climatic events that may have contributed to the high recovery. Research reported in Section 6 used spore traps in a number of different climatic zones to monitor inoculum dispersal in vineyards and also attempts to correlate dispersal patterns with weather conditions.

In Wagga Wagga during 2013-14, recovery of *D. seriata* from non-inoculated controls for the late pruning was significantly higher than for all other pruning times in the first two years of the study. Furthermore, following the same pruning event, recovery of *D. seriata* was significantly lower for inoculations made on days 1 to 28 than at all other pruning times in the first two years. Recovery from the early and late pruning times for 2013-14 were also significantly higher than for the corresponding times in 2014-15. Analysis of the weather data for Wagga Wagga showed significantly greater cumulative rainfall totals for the seven days preceding each of the pruning times in 2013, compared with 2014. As for *E. lata,* spores of the Botryosphaeriaceae are released with the onset of rain and are spread by wind or rain splash to infect exposed wound surfaces (Pitt *et al.* 2012). It is possible that the relatively high rainfall before each of the pruning events in 2013 led to the high recovery from the non-inoculated controls, although the reason for the very high recovery from the late pruning non-inoculated controls in 2013 is unclear.

The high recovery of *D. seriata* from non-inoculated controls across the first two pruning seasons, indicates the high level of natural disease pressure in the vineyard, compared with the other pathogens in this study, and hence the importance of wound protection to manage botryosphaeria dieback. In 2015-16, recovery of *N. luteum* across the pruning season was considerably lower than that of *D. seriata*, more closely reflecting results with *E. lata* in McLaren Vale. These results indicate that trunk disease pathogens vary in their prevalence of inoculum, and in response to the high levels of background infection by *D. seriata*, the less prevalent *N. luteum* was used in the third year of the Wagga Wagga trial. This species produces a yellow pigment in culture, which diminishes after four to five days, allowing for differentiation from *D. seriata* cultures which do not produce the pigment (Section 5.1). *N. luteum* was not recovered from any non-inoculated control vines, eliminating the confounding issues caused by background infection when using *D. seriata*.

Van Niekerk *et al.* (2011a) discussed the reasons for reduction observed in the susceptibility of grapevine wounds to infection by trunk disease pathogens, namely the mechanisms involved in wound repair. Sun *et al*. (2006) reported that grapevines start to develop tyloses in the exposed xylem vessels soon after canes are pruned, continuing until almost all xylem vessels are blocked after a week. In other woody plants, where tyloses develop in response to wounding, lignified and/or suberised secondary walls subsequently develop, eventually forming impenetrable layers in the xylem vessels (Biggs, 1987). Biggs (1990) showed that at higher average temperatures, the rate of suberisation in wounded bark is greater than at lower temperatures. Munkvold and Marois (1993, 1995) reported similar observations for grapevines, where higher mean temperatures after pruning correlated to increased rate of suberin accumulation in the pruning wounds as well as the rate of colonisation of pruning wounds by naturally occurring non-pathogenic micro-organisms, which contribute to reduced infection of wounds by *E. lata*. In McLaren Vale, the recovery of *E. lata* from the day 1 inoculations from the early and late pruned vines in 2013 and mid and late pruned vines in 2015 was similar, despite an increase in temperature and solar radiation over those times. This suggests that, at McLaren Vale, temperature did not affect the susceptibility of the pruning wounds to infection.

To the authors' knowledge, the DCA experiments are the first to compare duration of wound susceptibility of a range of grapevine cultivars to eutypa and botryosphaeria dieback pathogens. Whilst all cultivars could be infected by *E. lata* even 14 days after wounding, recovery at that time was less than half that of the day 1 inoculations, confirming the findings of the field wound susceptibility experiments conducted on Shiraz vines. For botryosphaeria dieback, all cultivars remained highly susceptible to infection by *N. luteum*, even 14 days after wounding. These results show that there was little difference in susceptibility in the six most commonly planted cultivars in Australia, and confirm that pruning wound protection strategies should be employed, regardless of the grape cultivar.

Most previous research in this area, including the current study, has evaluated wound susceptibility by artificial inoculation with extremely high doses of fungal spores. According to research conducted on apricots by Carter and Moller (1971), the estimated number of spores that would naturally infect a pruning wound was in the order of 10 spores. In the current and earlier research, 50-100 times this spore dose was used, representing an extreme disease pressure not normally exhibited on pruning wounds in vineyards. Elena *et al.* (2015) showed that applying fewer *E. lata* spores (as few as 10) resulted in reduced pathogen recovery, more closely reflecting natural disease pressure. Ayres *et al.* (2016) showed that using fewer spores (200) resulted in increased efficacy of preventative fungicide treatments when compared with higher spore doses. Future research will include Australian wine regions with different climates, and will aim to determine susceptibility of wounds to infection with lower spore doses, more closely reflecting that of natural conditions.

These results suggest that, in McLaren Vale and Wagga Wagga, there may be little advantage in choosing one pruning time over another, in terms of minimising the risk of infection by the trunk disease pathogens evaluated here. However, results highlight the importance of protecting pruning wounds for at least two weeks post-pruning, and the curative and preventative qualities of protective fungicides are reported in Section 8.2.

8 PRUNING WOUND PROTECTION

8.1 Introduction

Previous research (CRV 03/06S, SAR 06/01, Sosnowski *et al*. 2008, 2013) showed that treating pruning wounds with the benzimidazole fungicides benomyl and carbendazim prevented infection by *E. lata*. Carbendazim was also effective at reducing infection by *D. seriata* and *D. mutila* (Pitt *et al*., 2012). However, these fungicides were withdrawn from use on grapevines in 2001 and 2010, respectively. With this, and the increased awareness of the threat posed by grapevine trunk diseases in Australia (Sosnowski and Wicks 2012), new pruning wound treatments that control eutypa dieback and botryosphaeria dieback were identified, in particular fluazinam, tebuconazole and pyraclostrobin (SAR 1001, Pitt *et al.* 2012, Ayres *et al.* 2014), with subsequent label registrations for use of Emblem (fluazinam) and Gelseal (tebuconazole) following pruning. This supplemented previous registrations of paste products such as Greenseal (tebuconazole) and Garrison (cyproconazole plus iodocarb) which can only be applied by hand. Sosnowski *et al.* (2013b) and Ayres *et al.* (2014, 2016; SAR 1001) also demonstrated the use of commercial sprayers to apply fungicide treatments to pruning wounds effectively.

The aim of this study was to evaluate the critical timing of application for fluazinam, tebuconazole and pyraclostrobin, in order to determine how quickly the treatments must be applied after pruning and infection (curative) and then the period of efficacy following application (preventative). This information will ensure optimal wound protection against infection by both eutypa and botryosphaeria dieback (ED and BD) pathogens.

The second aim of this study was to identify and evaluate potential alternative products for wound protection. Polymer and silicate products which might provide a physical barrier to infection were evaluated, along with potassium bicarbonate and difenconazole, which were all compared with tebuconazole for efficacy against ED and BD pathogens.

8.2 Timing of application

8.2.1 Methods

Trials were established in 2013, 2014 and 2015 on Shiraz vines planted in 1996 (McLaren Vale Visitor Centre vineyard, SA) and Cabernet Sauvignon vines planted *circa* 1990 (Charles Sturt University vineyard, Wagga Wagga, NSW). In each trial, one-year-old canes were pruned to two buds, using secateurs, on 2, 1 and 14 July (SA) and 26, 24 and 30 July (NSW) in 2013, 2014 and 2015, respectively.

To evaluate curative control, on the day of pruning, wounds were moistened by spraying with sterile distilled water (SDW) immediately prior to inoculation with approximately 500 spores of *E. lata* (SA) or 1000 spores of *D. seriata* (2013) or *N. luteum* (2014 and 2015; NSW) suspended in 20 µL SDW and applied using a pipette. Using a paintbrush, wounds were treated with Cabrio (250 g a.i. pyraclostrobin /L; BASF) at 2 mL/L, Emblem (500 g a.i fluazinam /L; Cropcare Australasia) at 5 mL/L or Folicur (430 g a.i. tebuconazole /L; Bayer CropScience) at 1.5 mL/L water at 1, 3 or 6 days after pruning and inoculation. Controls, positive (inoculated) and negative (non-inoculated) were treated with SDW. Each treatment was allocated to a vine with 10 pruned canes. The trials were set up as randomised block designs with 10 replications.

In the same trials, to evaluate preventative control, using a paintbrush, wounds were treated with Cabrio at 2 mL/L, Emblem at 5 mL/L or Folicur at 1.5 mL/L water on the day of pruning, and then 1, 6 and 14 days after pruning and treatment, wounds were moistened by spraying with SDW immediately prior to inoculation with approximately 500 spores of *E. lata* (SA) or 1000 spores of *D. seriata* (2013) or *N. luteum* (2014 and 2015; NSW) suspended in 20 µL SDW and applied using a pipette. Controls, positive (inoculated) and negative (non-inoculated) were treated with SDW. Each treatment was allocated to a vine with 10 pruned canes. The trials were set up as randomised block designs with 10 replications.

Eutypa lata ascospores and *Diplodia seriata* and *Neofusicoccum luteum* spores were obtained using the methods described in Section 7.2.1

For each trial, canes were harvested from vines between April and May, 10-11 months after establishment 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 for ED and 2 minutes for BD and then washed twice in SDW. Using 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 in each plastic Petri dish filled with PDA amended with antibiotic (streptomycin sulfate 100mg/L for ED and chloramphenicol 100mg/L for BD), with two plates used for each cane for ED experiments. For BD experiments one plate was used for each cane and these were cut into four segments. Plates were incubated at 25°C under fluorescent light for 12 h each day for 7 days and then assessed for presence or absence of mycelial growth of *E. lata* (SA), *D. seriata* or *N. luteum* (NSW). Efficacy was based on the mean percent recovery (MPR) of *E. lata*, *D. seriata* or *N. luteum* from the treated canes. 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.

Climate data for McLaren Vale were provided by Measurement Engineering Australia (MEA) and the McLaren Vale Grape, Wine and Tourism Association, from an on-site automatic weather station (AWS) approximately 30 metres from the trial site. Climate data for Wagga Wagga were obtained from an AWS located 1.5 km from the trial site (www.bom.gov.au).

8.2.2 Results

8.2.2.1 Eutypa dieback

Curative control
Eutypa lata was recovered from 58, 22 and 75% of inoculated control canes and 3, 16 and 17% of naturally infected canes in 2013, 2014 and 2015 trials, respectively (Figure 8.1). When wounds were inoculated on the day of pruning and fungicides were applied at 1, 3 or 6 days later, all three products significantly reduced recovery of *E. lata* to between 5 and 26% in 2013 (Figure 8.1a). In 2014, recovery of *E. lata* was between 8 and 15% for canes treated with Cabrio, 25 and 42% for Emblem and 9 and 17% for Folicur (Figure 8.1b). In 2015, all three products reduced recovery of *E. lata* to between 19 and 58%, although reduction in recovery was less for Cabrio and Emblem than for Folicur (Figure 8.1c).

Preventative control
In the 2013 trial, when wounds were inoculated at 1, 6 and 14 days after pruning, *E. lata* was recovered from 60, 44 and 8% of inoculated control canes, respectively (Figure 8.2a). When inoculated at one day after application, all three fungicides significantly reduced recovery of *E. lata*. However, only Folicur significantly reduced recovery at six days after application, compared with inoculated controls.

In the 2014 trial, when wounds were inoculated at 1, 6 and 14 days after pruning, *E. lata* was recovered from 19, 11 and 22% of inoculated control canes, respectively (Figure 8.2b). When inoculated at 1, 6 and 14 days after application, *E. lata* was recovered from between 6 and 16% of canes treated with Cabrio, 25 and 38% with Emblem and 11 and 13% with Folicur. During laboratory assessments, plates were often overgrown with contaminants identified via molecular analysis as *Cryptovalsa ampelina,* which made identification of *E. lata* difficult.

In the 2015 trial, when wounds were inoculated at 1, 6 and 14 days after pruning, *E. lata* was recovered from 72, 62 and 27% of inoculated control canes, respectively (Figure 8.2c). When inoculated at 1 and 6 days after application, all three fungicides reduced recovery of *E. lata* to between 31 and 53%. However, none of the fungicides reduced recovery at 14 days after application, compared with inoculated controls.

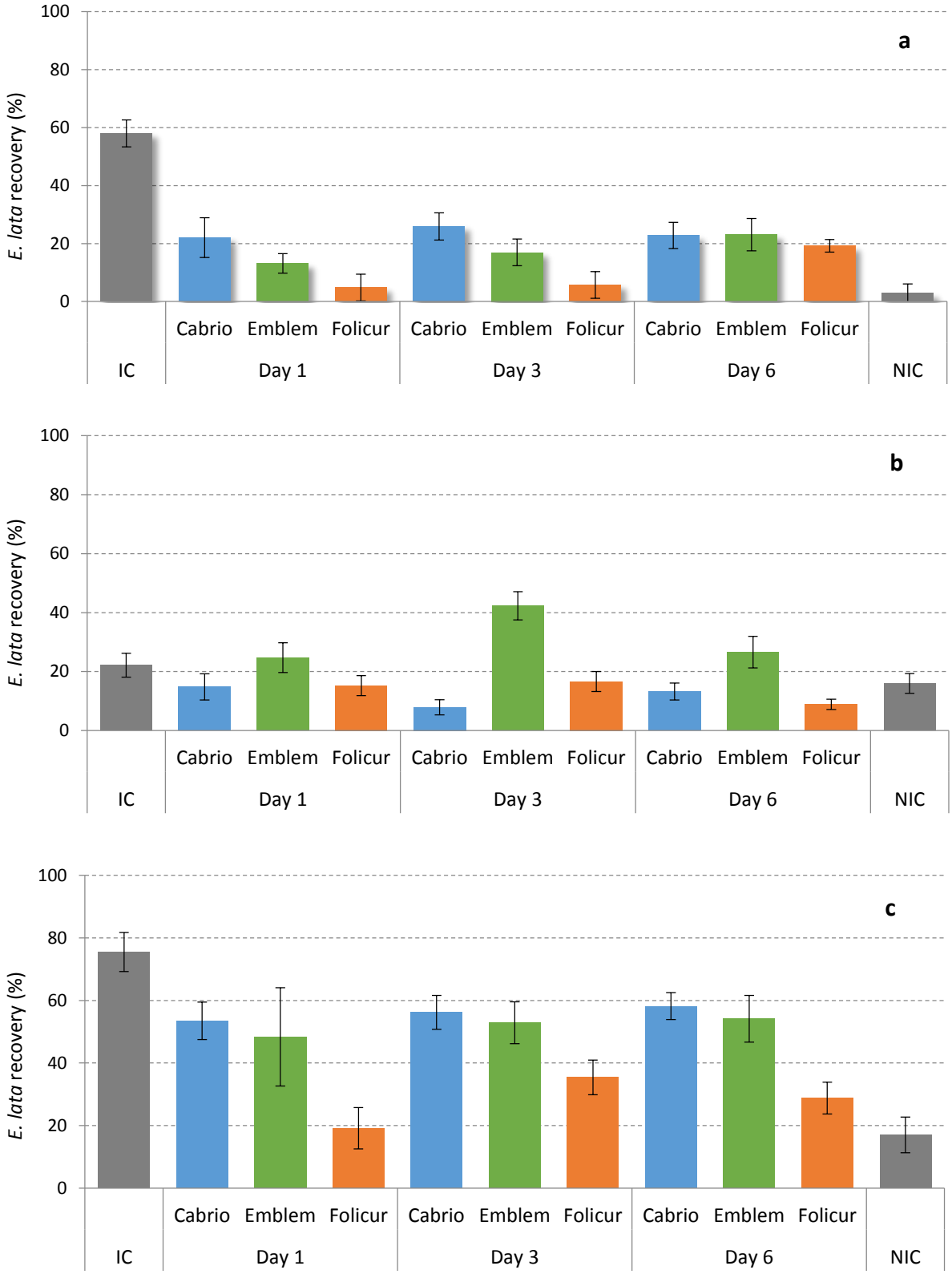


Figure 8.1 Eutypa dieback curative control. Incidence of *Eutypa lata* recovery from canes which were inoculated with 500 spores on the day of pruning (a - 2 July 2013, b – 1 July 2014, c - 14 July 2015) and then fungicides were applied 1, 3 or 6 days after pruning. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

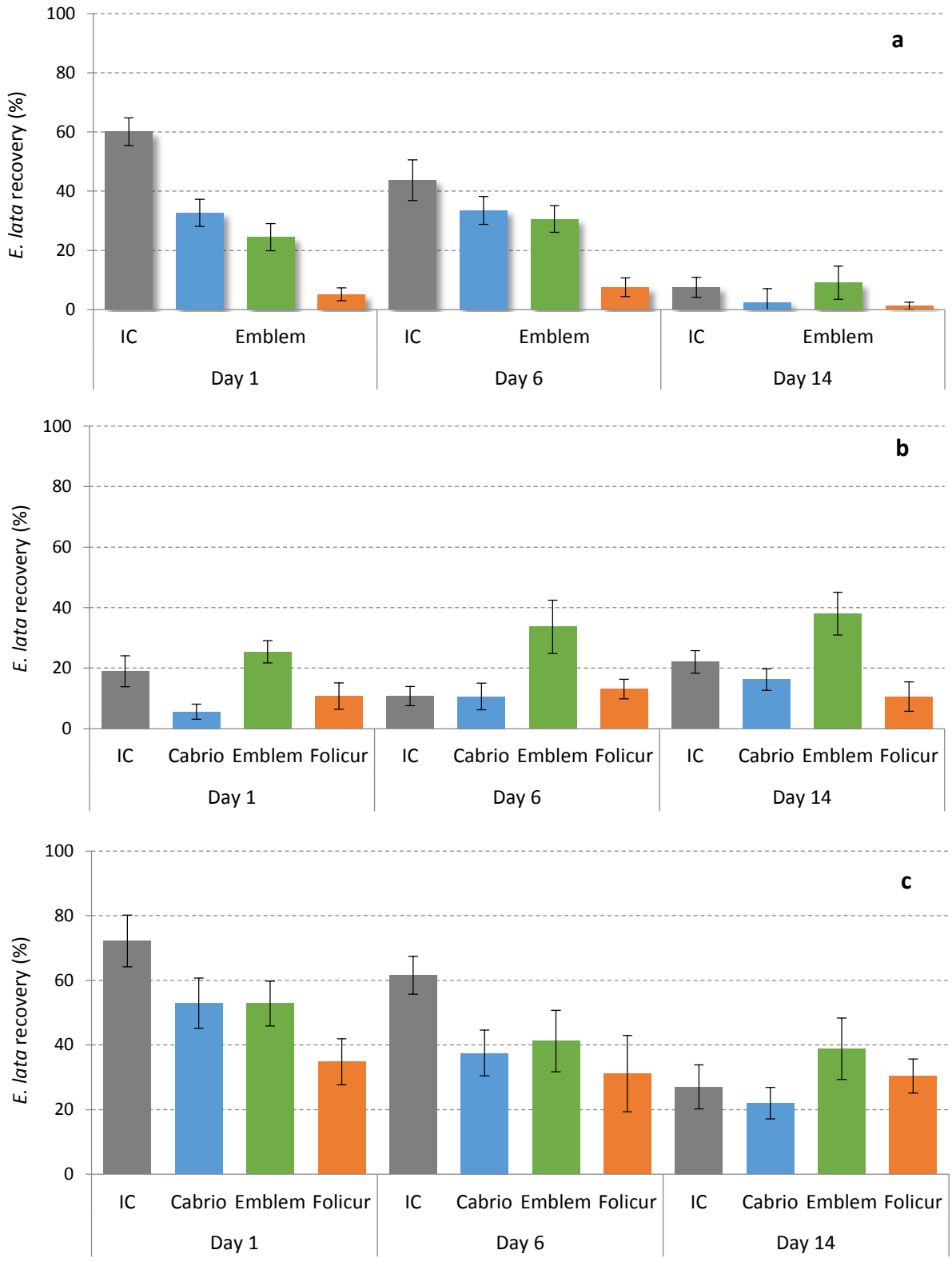


Figure 8.2 Eutypa dieback preventative control. Incidence of *Eutypa lata* recovery from canes with fungicides applied to wounds on the day of pruning (a - 2 July 2013, b – 1 July 2014, c - 14 July 2015) and then inoculated with 500 spores 1, 6 or 14 days after pruning. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

8.2.2.2 Botryosphaeria dieback

Curative control

In the 2013 trial, *D. seriata* was recovered from 100% of inoculated control canes and 28% of naturally infected canes (Figure 8.3a). When wounds were inoculated on the day of pruning and fungicides were applied one day later, recovery of *D. seriata* was reduced to 84, 70 and 54% by Cabrio, Emblem and Folicur, respectively. The three fungicides were less effective when applied three and six days after inoculation, with recoveries ranging between 75 and 100%.

Neofusicoccum luteum was recovered from 88 and 78% of inoculated control canes in 2014 and 2015, respectively, but was not recovered from any non-inoculated control canes (Figure 8.3b-c). When wounds were inoculated on the day of pruning and fungicides were applied at 1, 3 or 6 days later, all three products significantly reduced recovery of *N. luteum* to between 4 and 54% (2014) and 0 and 38% (2015).

Preventative control

In the 2013 trial, when wounds were inoculated at 1, 6 and 14 days after pruning, *D. seriata* was recovered from 100, 98 and 90% of inoculated control canes, respectively (Figure 8.4a). When inoculated one day after application, Folicur and Emblem significantly reduced recovery of *D. seriata* to 78 and 90%, respectively, while Cabrio was not effective, with 100% pathogen recovery. For all three fungicides, pathogen recovery six days after application was not significantly different from inoculated controls. However, 14 days after application, *D. seriata* recovery was significantly lower than the inoculated controls with 28, 46 and 60% recovery for Cabrio, Emblem and Folicur, respectively.

In the 2014 trial, when wounds were inoculated 1, 6 and 14 days after pruning, *N. luteum* was recovered from 74, 80 and 46% of inoculated control canes respectively, and all three fungicides significantly reduced recovery of *N. luteum* to between 0 and 24%. (Figure 8.4b).

In the 2015 trial, when wounds were inoculated at 1, 6 and 14 days after pruning, *N. luteum* was recovered from 82, 72 and 36% of inoculated control canes, respectively and all three fungicides significantly reduced recovery of *N. luteum* to 2 to 46% (Figure 8.4c).

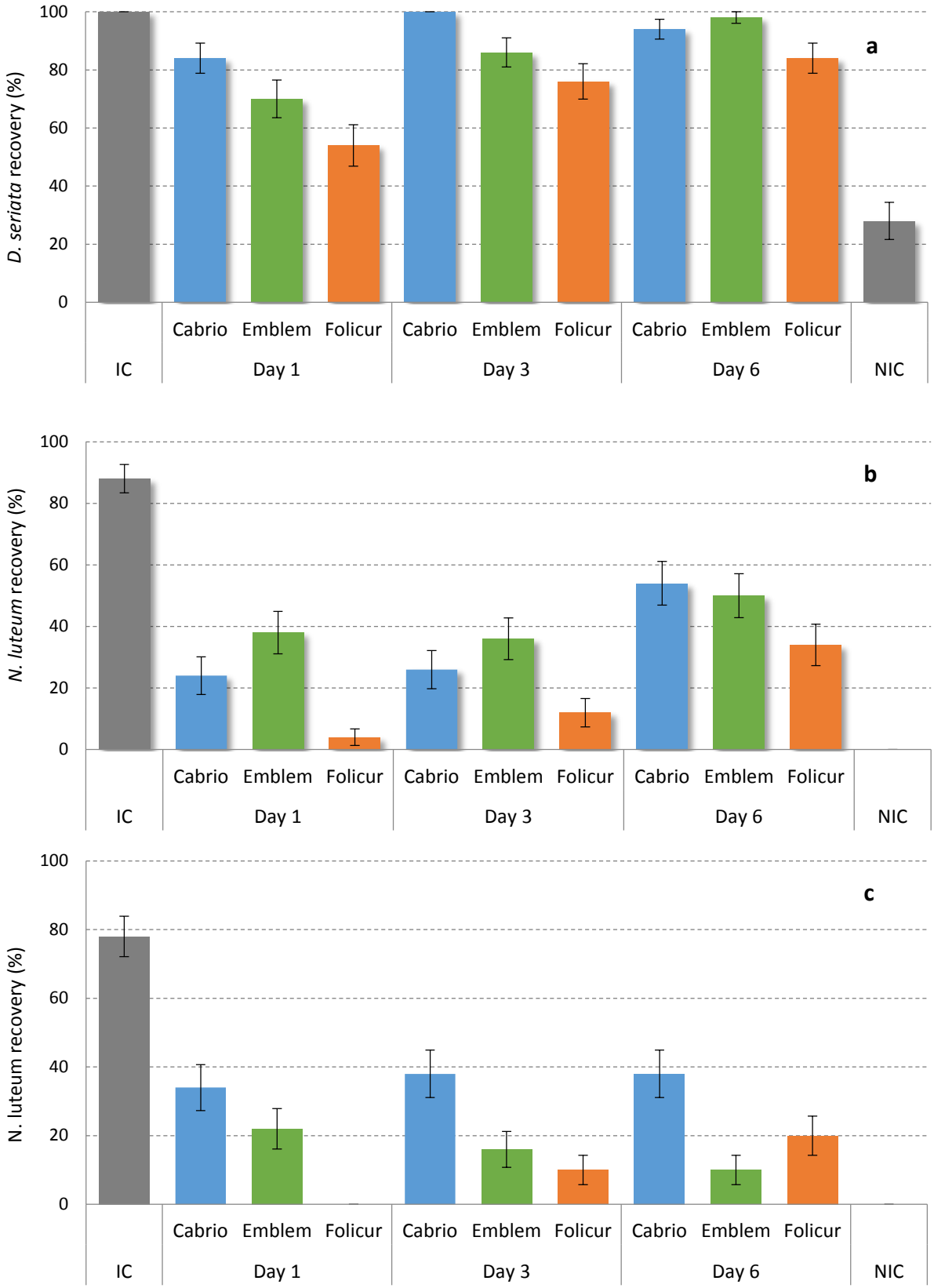


Figure 8.3 Botryosphaeria dieback curative control. Incidence of *Diplodia seriata* and *Neofusicoccum luteum* recovery from canes which were inoculated with 1000 spores on the day of pruning (a - 26 July 2013, b - 24 July 2014, c - 30 July 2015) and then fungicides were applied 1, 3 or 6 days after pruning. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

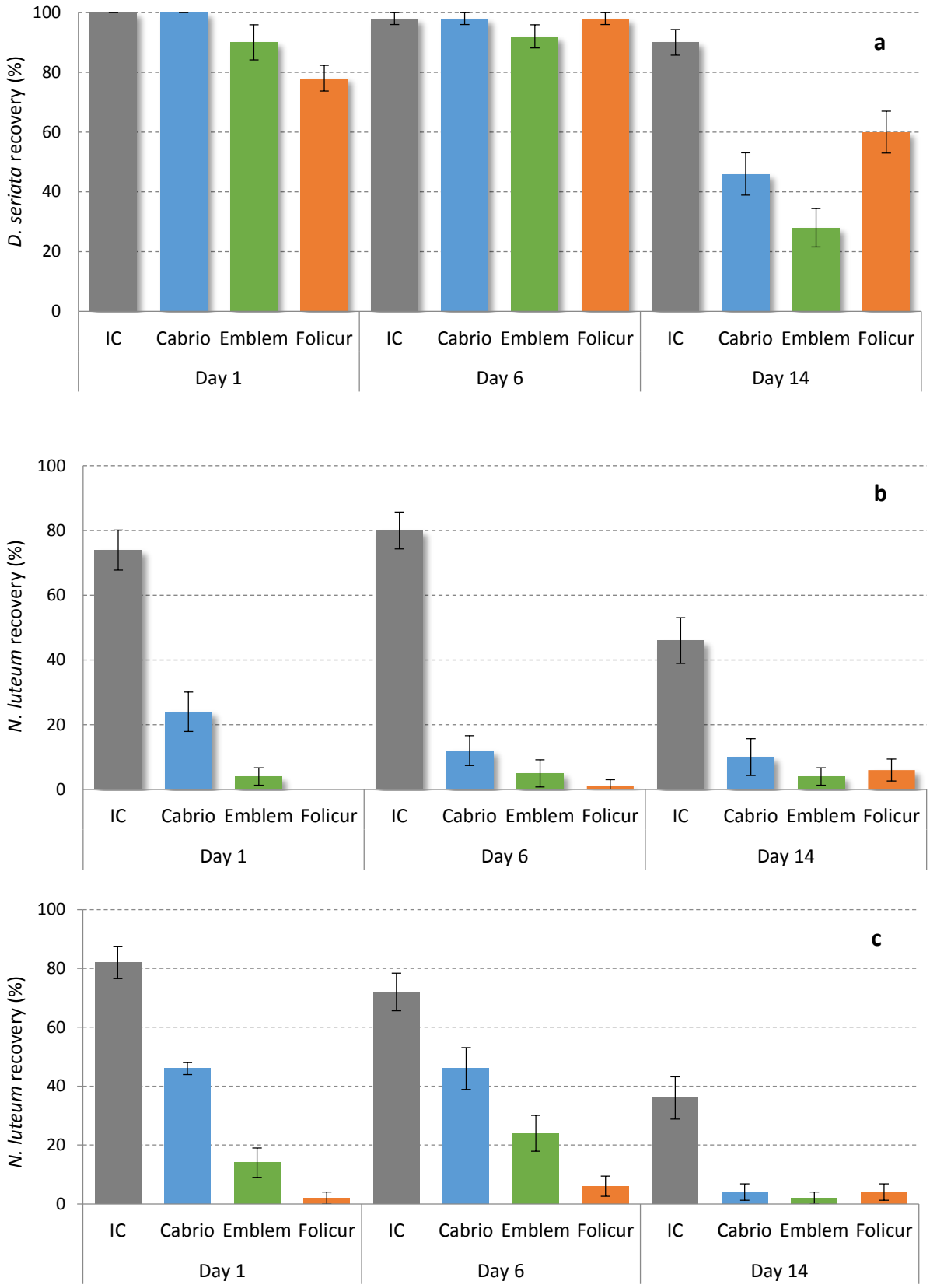


Figure 8.4 Botryosphaeria dieback preventative control. Incidence of *Diplodia seriata* and *Neofusicoccum luteum* recovery from canes with fungicides applied to wounds on the day of pruning (a - 26 July 2013, b - 24 July 2014, c - 30 July 2015) and then inoculated with 1000 spores 1, 6 or 14 days after pruning. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

8.2.2.3 Climate data

The climate data for McLaren Vale were examined to assess correlations with *E. lata* recovery data in order to explain unusual patterns (Table 8.1). It revealed there was less rainfall in the seven days preceding pruning in 2013 than in 2014 and 2015, with 4, 11 and 21 mm for each of the years, respectively. There was a similar pattern for the day 1 inoculation with 4, 11 and 31 mm for 2013, 2014 and 2015, respectively. Most other parameters were similar between years with the exception of higher temperature in 2015 at pruning, with a maximum of 22.3°C compared with 17.1 and 14.4°C for 2013 and 2014, respectively, and higher average solar radiation at, and for two days after, pruning in 2013 than in 2014 and 2015; 90 and 85, 74 and 75, and 73 and 65 W/m^2 in 2013, 2014 and 2015, respectively.

The climate data for all years of the Wagga Wagga trials showed the lowest rainfall in the seven days preceding pruning was in the third year of trials, with 9.8, 9.6 and 1.2 mm recorded for 2013, 2014 and 2015, respectively, prior to the preventative pruning, and 6.4, 4.2 and 1.2 mm for the same years, respectively, for prior to the curative pruning. The second year of trials had the lowest temperatures at the time of preventative pruning, with 17.6, 10.4 and 14.9°C for 2013, 2014 and 2015, respectively, but, for the curative pruning time, the third year was coolest, with 16, 13.7 and 11.9°C recorded for 2013, 2014 and 2015, respectively. Total solar radiation was higher at, and for two days after, preventative and curative pruning in 2015 than 2013, with 2.8 and 2.6, and 2.3 and 2.6 kWh/m^2 recorded for preventative and curative pruning, respectively, in 2015, compared with 1.3 and 1.5, and 2.0 and 2.0 kWh/m^2 for the same times in 2013.

Table 8.1 *Eutypa lata* recovery (%) and weather data corresponding to treatment dates for the pruning wound protection trials conducted at McLaren Vale in 2013, 2014 and 2015. Parameters include maximum temperature (°C), Average relative humidity (%) and average daily solar radiation (W/m^2) the day of treatment and 1, 2, 7 and 14 days following treatment, and cumulative rainfall for 2 and 7 days prior to treatment plus the day of treatment and 2, 7 and 14 days following treatment. Colour coding reflects relative value.

		E. lata recovery (%)		Max temp. (°C)				Ave relative humidity (%)				Ave solar radiation (W/m^2)				Cumulative rainfall (mm)					
Treatment	Date	Inoc	NIC	1d	2d	7d	14d	1d	2d	7d	14d	1d	2d	7d	14d	-7d	-2d	1d	2d	7d	14d
prune	2/07/2013	58	3	17.1	16.7	15.1	16.0	87	81	78	79	90	85	77	78	4	4	0	0	16.8	41.2
day 1	3/07/2013	60		16.2	16.2	14.7	15.8	75	68	78	80	80	95	80	77	4	0	0	0	16.8	42.6
day 3	5/07/2013			14.5	14.1	14.7	16.1	70	73	80	81	38	61	82	74	7	3	3.2	10	16.8	57.6
day 6	8/07/2013	44		14.2	14.3	16.6	15.9	88	87	81	81	85	97	82	74	17	7	0.4	0.4	21.8	61
day 14	14/07/2013	8		18.6	17.3	16.3	15.4	79	84	82	80	45	56	60	73	22	21	0.4	3.4	37.2	66.6
prune	1/07/2014	22	16	14.4	14.6	14.8	14.2	89	90	81	78	74	75	83	78	11	4	3	3	13.8	41.8
day 1	2/07/2014	19		14.7	15.3	14.8	14.0	91	86	76	77	75	83	83	77	11	3	0	0	10.8	39.4
day 3	4/07/2014			15.1	14.8	14.2	13.8	69	69	72	76	107	89	73	75	8	0	0	2.6	23.8	56.8
day 6	7/07/2014	11		15.1	14.8	13.8	13.4	81	67	75	78	99	86	72	72	14	8	0	0	28	51.6
day 14	15/07/2014	22		12.1	12.2	13.2	14.5	71	78	80	79	60	55	71	78	29	1	0.6	3.2	23.6	45.6
prune	14/07/2015	75	17	22.3	20.4	16.5	17.5	86	85	83	79	73	65	81	87	21	6	3.4	14.8	20	37.4
day 1	15/07/2015	72		18.5	15.8	15.1	17.2	84	85	80	79	57	64	86	89	31	15	11.4	16.2	16.6	34.4
day 3	17/07/2015			18.6	15.2	14.8	17.5	83	84	77	77	80	67	89	91	36	5	0.4	0.4	3	18.2
day 6	20/07/2015	62		13.1	13.0	17.3	17.1	79	71	75	77	116	114	93	85	20	0	0	0	16.2	25.6
day 14	28/07/2015	27		18.3	19.2	15.7	15.2	86	85	79	80	110	103	76	85	18	2	0.4	0.4	8.8	14.6

Table 8.2 Recovery percentage of *Diplodia seriata* (2013) and *Neofusicoccum luteum* (2014 and 2015) and weather data corresponding to treatment dates for the pruning wound protection trials conducted at Wagga Wagga. Parameters include maximum temperature (°C) and total daily solar radiation (kW/m^2*4) on the day of treatment and 1, 2, 7 and 14 days following treatment, and cumulative rainfall for 2 and 7 days prior to treatment plus the day of treatment and 2, 7 and 14 days following treatment. Colour coding reflects relative value level.

		D. seriata and N. luteum recovery (%)		Max temp (°C)				Total solar radiation (kWh m2)				Cumulative rainfall (mm)					
Treatment	Date	Inoc	NIC	1d	2d	7d	14d	1d	2d	7d	14d	-7d	-2d	1d	2d	7d	14d
preventative prune	25/06/2013			17.6	17.6	15.6	14.9	2.5	2.6	2.1	2.3	9.8	6.4	6.4	6.4	6.4	8
preventative d1	26/06/2013	100		17.6	16.8	15.4	14.6	2.6	2.5	2.1	2.3	6.4	6.4	0	0	0	1.6
curative pruning	27/06/2013	100	28	16	15.9	15	14.4	2.3	2.0	2.1	2.3	6.4	0	0	0	0.2	1.6
curative d1	28/06/2013			15.7	15.4	14.9	14.3	1.6	1.8	2.0	2.3	6.4	0	0	0	0.2	1.6
curative d3	30/06/2013			15.4	13.7	14.2	14.4	2.6	2.0	2.3	2.4	6.4	0	0	0	0.6	2
preventative d6	1/07/2013	98		11.9	14.2	13.9	14.3	1.3	2.0	2.2	2.3	6.4	0	0	0	0.6	2
curative d6	3/07/2013			14.3	15.1	13.7	14.4	2.4	2.1	2.5	2.1	0.2	0.2	0.2	0.2	1.6	11.6
preventative d14	9/07/2013	90		12.9	14	14.6	15	2.7	2.7	2.0	2.1	1.6	1	0	0	8.2	29.2
preventative prune	1/07/2014			10.4	10.6	10.6	11.3	1.3	1.5	1.7	1.9	9.6	5.6	1	1.2	5.2	9.6
preventative d1	2/07/2014	74		10.7	9.65	11	11.5	1.6	1.6	1.8	1.9	5.8	1.2	0.2	0.6	4.2	8.6
preventaive d6	7/07/2014	80		11.3	12.5	11.9	12.3	1.9	2.0	2.0	2.2	5.2	3.4	3.4	3.4	7.8	22.2
curative prune	8/07/2014	88	0	13.7	13.2	12.1	12.5	2	2.0	2.1	2.3	4.2	3.4	0	0	4.4	18.8
curative d1	9/07/2014			12.6	11.5	11.9	12.5	1.9	1.9	2.0	2.4	4	0	0	1.2	4.4	18.8
curative d3	11/07/2014			11.4	11.4	12.5	12.9	2	1.8	2.1	2.4	5.2	1.8	0.6	0.6	12.2	17.6
curative d6	14/07/2014			13.1	12.6	12.8	13.7	2.7	1.8	2.4	2.5	4.4	2.6	0	0	14.4	14.6
preventative d 14	15/07/2014	46		12.1	13.5	12.8	13.8	0.9	1.6	2.4	2.5	4.4	0	0	7.4	14.4	14.6
preventative prune	29/06/2015			14.9	12.5	11.3	12.3	2.8	2.6	2.4	2.4	1.2	0	0	0	1.2	21.2
preventative d1	30/06/2015	82		10.1	9.8	10.9	12	2.3	2.3	2.4	2.3	1.2	0	0	0	1.2	25.8
preventative d6, curative prune	6/07/2015	72, 78	0	11.9	13.1	13.2	12.1	2.3	2.6	2.3	2.2	1.2	0	0	0.4	20	36.4
curative d1	7/07/2015			14.2	14.4	13.1	12.3	2.8	2.9	2.2	2.3	1.6	0.4	0.4	0.4	24.6	36.4
curative d3	10/07/2015			14.6	14	11	12.5	2.6	1.8	1.6	2.2	6.6	6.2	6.2	6.2	36	53.8
curative d6	13/07/2015			11	9.8	11	12.6	1.2	1.7	2.1	2.2	24.6	18	4.6	4.6	16.4	37.8
preventative d14	14/07/2015	36		8.6	8.3	11.6	12.5	2.2	1.7	2.4	2.3	24.2	4.6		5.8	11.8	34.2

8.3 Alternative products

8.3.1 Methods

Grapevine canes (cvs. Shiraz in SA and Cabernet Sauvignon in NSW) were used in a detached cane assay, using methods similar to those described in Section 7.3.1.

In the first experiment, the treatments; Digger (250g a.i. difenoconazole /L; Nufarm) at 0.25, 0.75 and 2.5 mL/L, Potassium silicate (w/v 17.3% silicon and 15.3% potassium; Nutri-Tech Solutions) at 3 and 30 mL/L, Sudo Shield (*Pseudomonas fluorescens*; Nutri-Life), Droughtshield (spray polymer; Yates) and Folicur at 0.3 mL/L water, were each applied by paintbrush to wounds. On the following day, wounds were inoculated with 200 spores of *E. lata* (SA) or *N. luteum* (NSW). Untreated controls were either inoculated with the spore suspension (positive control) or not inoculated (negative control). The experiments were repeated.

In a second experiment, HML 32 (170 g/L fatty acids (potassium salts) and 264 g/L potassium bicarbonate; Henry Manufacturing Ltd) at four concentrations: 100%, 10%, 5% and 2%, and Folicur at 0.3 mL/L water were applied by paintbrush to wounds. On the following day, wounds were inoculated with 200 spores of *E. lata* (SA) or *N. luteum* (NSW). Untreated controls were either inoculated with the spore suspension (positive control) or not inoculated (negative control). The experiments were repeated.

After four weeks, canes were processed for assessment as described in section 8.2.1. Efficacy was based on the mean percent recovery (MPR) of *E. lata* or *N. luteum* from the treated canes by isolation on PDA. 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.

8.3.2 Results

In the first experiment for eutypa dieback, *E. lata* was recovered from 97% of inoculated control canes but was not recovered from non-inoculated canes (Figure 8.5a). Folicur, when applied to wounds, significantly reduced recovery of *E. lata* to 25%. Application of Digger to wounds significantly reduced *E. lata* recovery to 75% at the two highest rates evaluated, but no other treatments reduced recovery compared with the inoculated control. In the repeated experiment, *E. lata* was recovered from 56% of inoculated control canes and was not recovered from non-inoculated canes (Figure 8.5b). Folicur, when applied to wounds, significantly reduced recovery of *E. lata* to 9%. The application of Droughtshield to wounds also significantly reduced *E. lata* recovery to 34%. No other treatments reduced recovery compared with the inoculated control.

In the first experiment for botryosphaeria dieback, *N. luteum* was recovered from 59% of inoculated control canes and there was 3% recovery from non-inoculated canes (Figure 8.6a). Digger (at 0.75 mL/L) and Potassium silicate (at 30 mL/L) significantly reduced *N. luteum* recovery to 28% but no other treatments reduced recovery compared with the inoculated control. In the repeated experiment, *N. luteum* was recovered from 81% of inoculated controls and was not recovered from the non-inoculated controls (Figure 8.6b). Folicur and Digger at 2.5 mL/L significantly reduced recovery to 31 and 50%, respectively, but no other treatments reduced recovery compared with the inoculated control.

In the second experiment for eutypa dieback, *E. lata* was recovered from 52% of inoculated control canes but was not recovered from non-inoculated canes (Figure 8.7a). Folicur application to wounds significantly reduced recovery to 6%, but none of the HML 32 treatments reduced recovery compared with the inoculated control. In the repeated experiment, *E. lata* was recovered from 34% of inoculated control canes but was not recovered from non-inoculated canes (Figure 8.7b). Folicur significantly reduced recovery to 6% and HML32 at 100% concentration significantly reduced *E. lata* recovery to 16%. None of the other treatments reduced the recovery of *E. lata* compared with the inoculated controls.

In the second experiment for botryosphaeria dieback, in July 2015, *N. luteum* was recovered from 81% of inoculated control canes and was not recovered from non-inoculated canes (Figure 8.8a). HML 32 at 5, 10 and 100% concentration significantly reduced recovery of *N. luteum* to 59, 59 and

29%, respectively, and Folicur significantly reduced recovery to 31%. HML 32 at 2% concentration did not reduce recovery of *N. luteum*, compared with the inoculated control. In the repeated experiment,

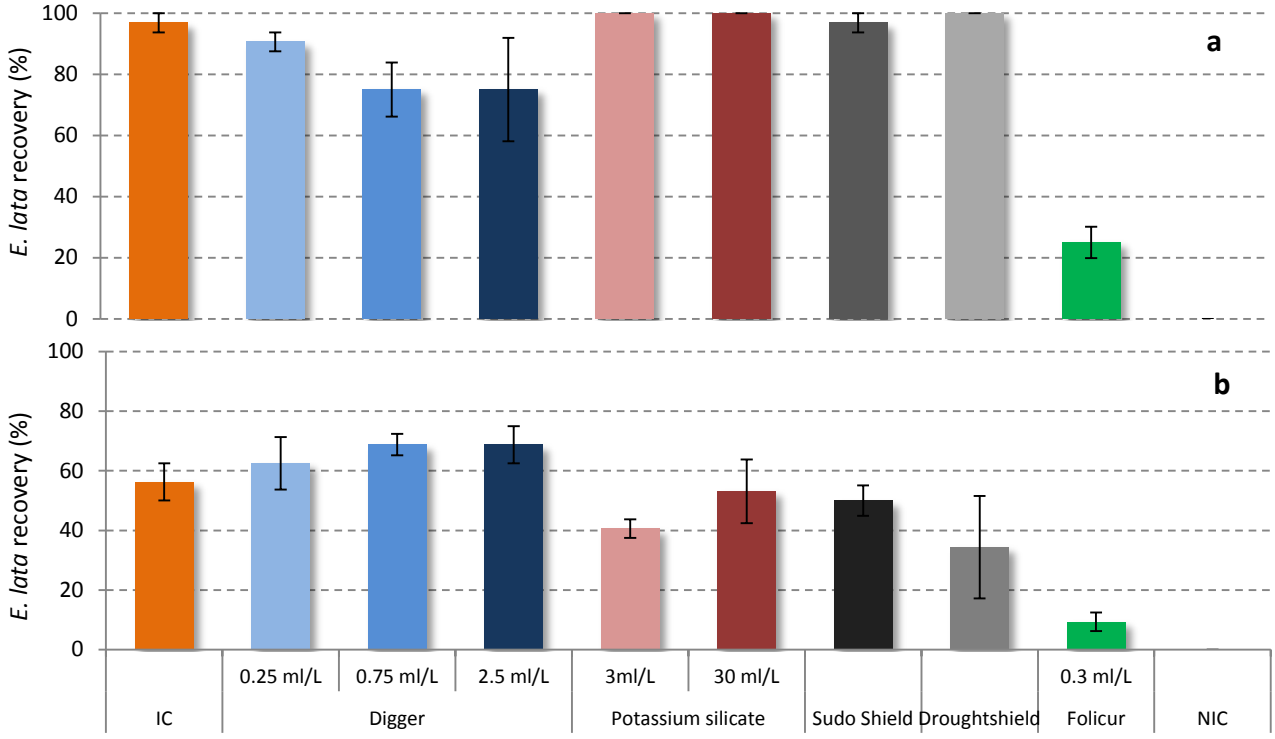


Figure 8.5 Incidence of *Eutypa lata* recovery from Shiraz canes with treatments applied to wounds on the day of pruning (a – 27 March 2014, b – 29 October 2015) and then inoculated with 200 spores on the following day. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

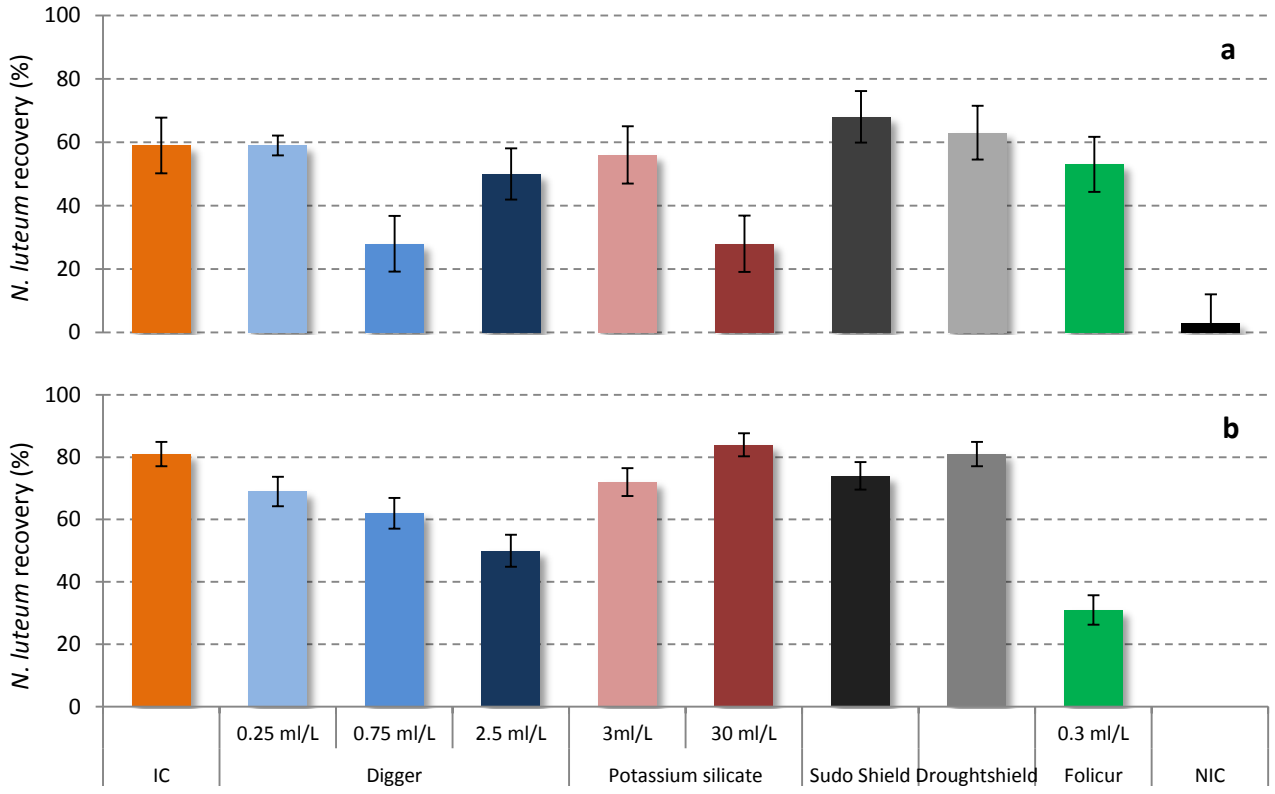


Figure 8.6 Incidence of *Neofusicoccum luteum* recovery from Cabernet Sauvignon canes with treatments applied to wounds on the day of pruning (a – 10 January 2014, b – 30 July 2015) and then inoculated with

200 spores on the following day. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

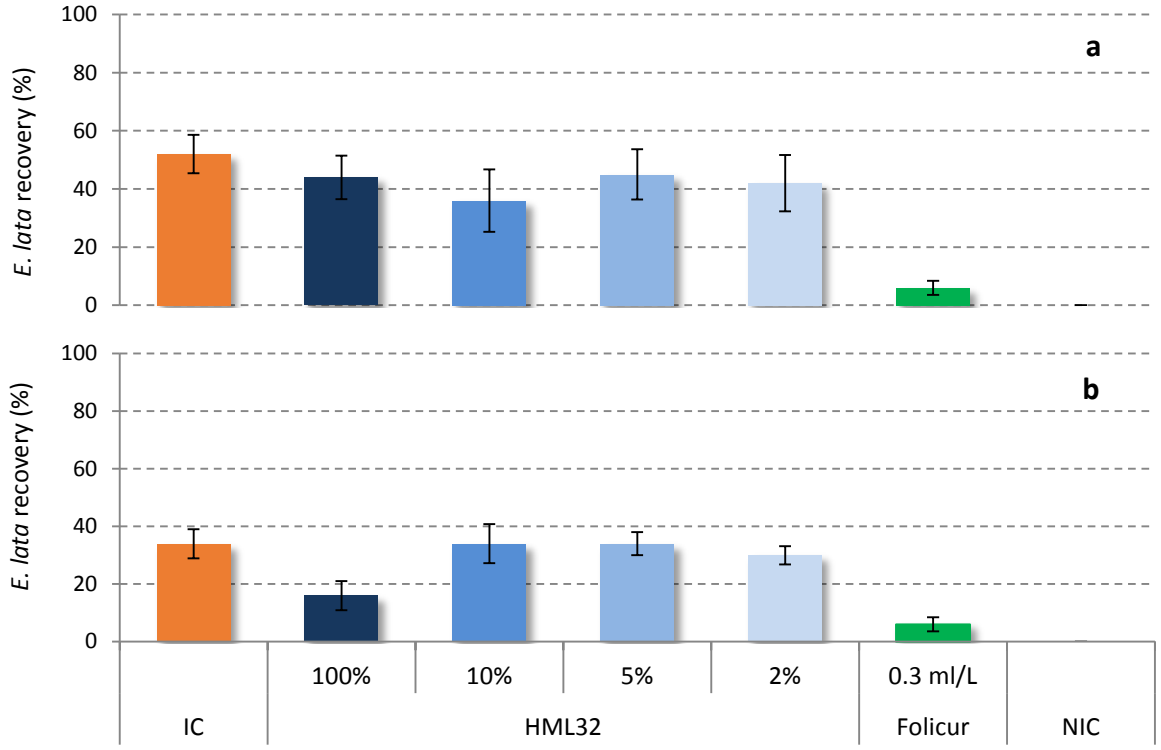


Figure 8.7 Incidence of *Eutypa lata* recovery from Shiraz canes with treatments applied to wounds on the day of pruning (a – 28 January 2015, b – 30 October 2015) and then inoculated with 200 spores on the following day. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

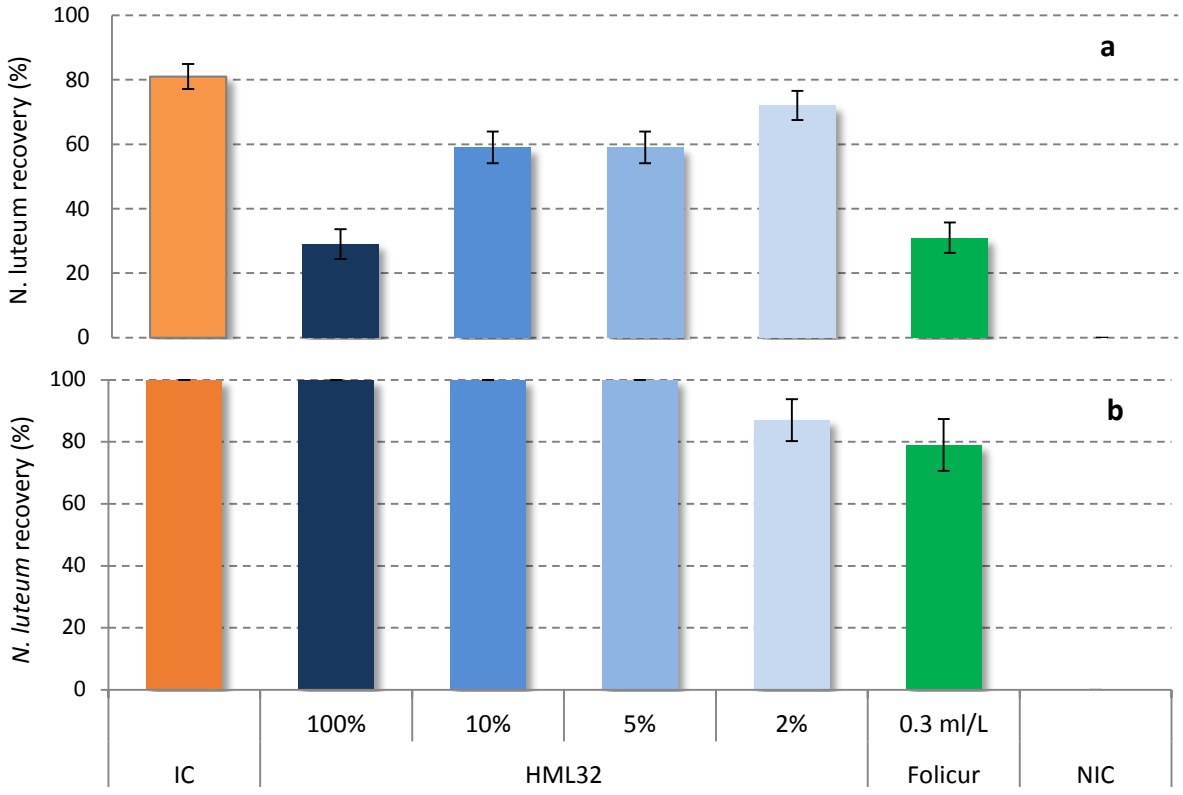


Figure 8.8 Incidence of *Neofusicoccum luteum* recovery from Cabernet Sauvignon canes with treatments applied to wounds on the day of pruning (a – 30 July 2015, b – 29 August 2016) and then inoculated with

200 spores on the following day. IC = inoculated control, NIC = non-inoculated control. Bars represent standard error of the mean.

N. luteum was recovered from 100% of inoculated control canes and was not recovered from the non-inoculated canes. Folicur significantly reduced recovery to 79% and HML 32 at 2% concentration significantly reduced recovery to 87%. None of the other treatments reduced recovery of *N. luteum* compared with the inoculated controls.

8.4 Discussion

Field trials were established to assess the timing of fungicide application relative to pruning, and showed that Cabrio, Emblem and Folicur exhibited curative properties when applied to pruning wounds for control of *E. lata* and *N. luteum*. Therefore a single application once a week may be sufficient to protect wounds, rather than each day following pruning activity in the vineyard. This is likely to improve logistics and, together with effective application of fungicides with commercial sprayers (Sosnowski *et al.* 2013b, Ayres *et al.* 2016), will encourage greater adoption of wound protection strategies for control of grapevine trunk disease.

All three fungicides provided preventative control of *N. luteum* for up to two weeks after application. All three fungicides controlled *E. lata* for up to a week, after which recovery from inoculated controls was significantly reduced due to natural wound healing, reducing the likelihood of infection.

Recovery of *E. lata* from the inoculated controls in the 2014 trials was very low compared to the 2013 and 2015 trials and was similar to the non-inoculated controls. Visual analysis of the climate data for McLaren Vale does not indicate any obvious climatic events that may have led to the low recovery. Furthermore, spore inoculum germination tests undertaken prior each inoculation revealed high germination for the suspension prepared for the inoculation in question (data not shown). The PDA plates from the laboratory assessment of canes from this trial were contaminated with *Cryptovalsa ampelina,* which made assessment difficult and confounded the results.

Recovery of *Diplodia seriata* was reduced by all three fungicides in the 2013 trial, but to a much lesser extent than *N. luteum* in the subsequent trials in Wagga Wagga. Recovery of *D. seriata* from inoculated controls was extremely high, which reflected disease pressure much greater than would occur in the vineyard, most likely leading to the reduced efficacy of fungicide treatments. In addition, background levels of infection were high as indicated by non-inoculated controls, also confounding results. For this reason, *N. luteum,* which produces a yellow pigment in culture, making it more readily distinguishable from *D. seriata* cultures which do not produce the pigment (see Section 5.1) was used in subsequent trials. Results with *N. luteum* more closely reflected those of *E. lata*.

Considering the challenges involved with conducting field trials with *D. seriata,* and the knowledge that a number of other fungal species are involved, it may be important to evaluate pruning wound protection with other species that cause BD. Further testing of *D. seriata* (the most prevalent species in Australia) at lower spore doses will also be necessary, to evaluate the efficacy of fungicides in preventing wound infection at closer to natural inoculum levels.

The recovery of *E. lata* from the inoculated control canes and many of the treated canes in 2015 was significantly greater than in 2013. Further, recovery from the non-inoculated control canes (reflecting natural inoculum levels) was relatively high in 2014 and 2015 at 16 - 17%, compared with just 3% in 2013. Carter (1957) showed that *E. lata* produces airborne spores that are released in wet weather. According to the McLaren Vale climate data, cumulative rainfall for the seven days prior to pruning and establishment of the fungicide trial was low in 2013 compared with that in the following years. Caesar and Pearson (1983) showed that UV radiation played a significant role in ascospore mortality, and Rotem *et al.* (1985) showed that solar radiation, even more than high temperatures, is a major factor in mortality of fungal spores. As solar radiation was greater in 2013 than in the following years, this may have led to reduced spore viability, possibly due to sensitivity of *E. lata* spores to UV light.

Although differences in rainfall, temperature and solar radiation were recorded in Wagga Wagga, with higher rainfall in the seven days prior to pruning, lower solar radiation when preventative and curative pruning were conducted, and lower temperature at preventative and higher temperature at curative pruning times in 2014, than 2015, this was not reflected in significant differences in recovery from the

inoculated controls. The reason for apparent lack of influence of climatic factors on recovery on *N. luteum* from inoculated canes should be a focus of future research.

These trials used very high inoculum spore doses that do not necessarily reflect natural disease pressure, as discussed in Section 7.4. It is therefore likely that if spore doses more closely reflected those that would occur naturally, the fungicides would provide greater disease control than was demonstrated here.

None of the alternative products assessed was able to consistently reduce infection by either pathogen. Nonetheless, future work will continue to assess alternative products, including chitosan and vanillin, for their ability to prevent infection by trunk disease pathogens.

Results of the susceptibility trials (Section 7) suggest that the most important period of wound susceptibility to trunk disease pathogens is 2-3 weeks post pruning. The results of these fungicide timing trials indicate that the three fungicides assessed can control *E. lata* and *N. luteum* when wounds are treated up to six days after infection, and will continue to provide control of both pathogens for 1-2 weeks. Therefore, if applied six days post-pruning, a single application could provide up to three weeks of wound protection.

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 more recently was demonstrated to control eutypa dieback (ED) in grapevines (Sosnowski *et al,* 2011b; SAR 06/01), with current recommendations suggesting to cut out all vine wood visibly affected by ED and an additional 10-20 cm of non-symptomatic wood. The infected wood should be burnt, buried or composted. Water shoot(s) produced from the vine stump are trained to replace the missing vine or cordon(s). In order to ascertain if remedial surgery could also be applied to manage botryosphaeria dieback (BD), field trials were established in three vineyards for two regions of New South Wales (NSW).

9.2 Methods

Remedial surgery trials were established in the Hunter Valley (two vineyards) and Harden (one vineyard) in October 2013 and November 2014, respectively. In the Hunter Valley, Vineyard 1 (HV1) comprised 24-year-old grafted Semillon vines and Vineyard 2 (HV2) comprised 20-year-old own-rooted Semillon vines. Vineyard 3 (H1) in Harden comprised 24-year-old own-rooted Shiraz vines. 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-b). Following assessment, the trunks were cut using hydraulic loppers (HV1; Figure 9.1c-d) or a chainsaw (HV2 and H1) either high (just below crown), mid (mid-point between ground and crown) or low (20 cm above graft union or ground), 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 (10 g/L tebuconazole) (Figure 9.2d-e). Sections of symptomatic wood were assessed for the presence of trunk disease fungi by isolating onto artificial media. The production of water shoots was recorded during the following growing seasons and the highest shoot was trained to form a new trunk.

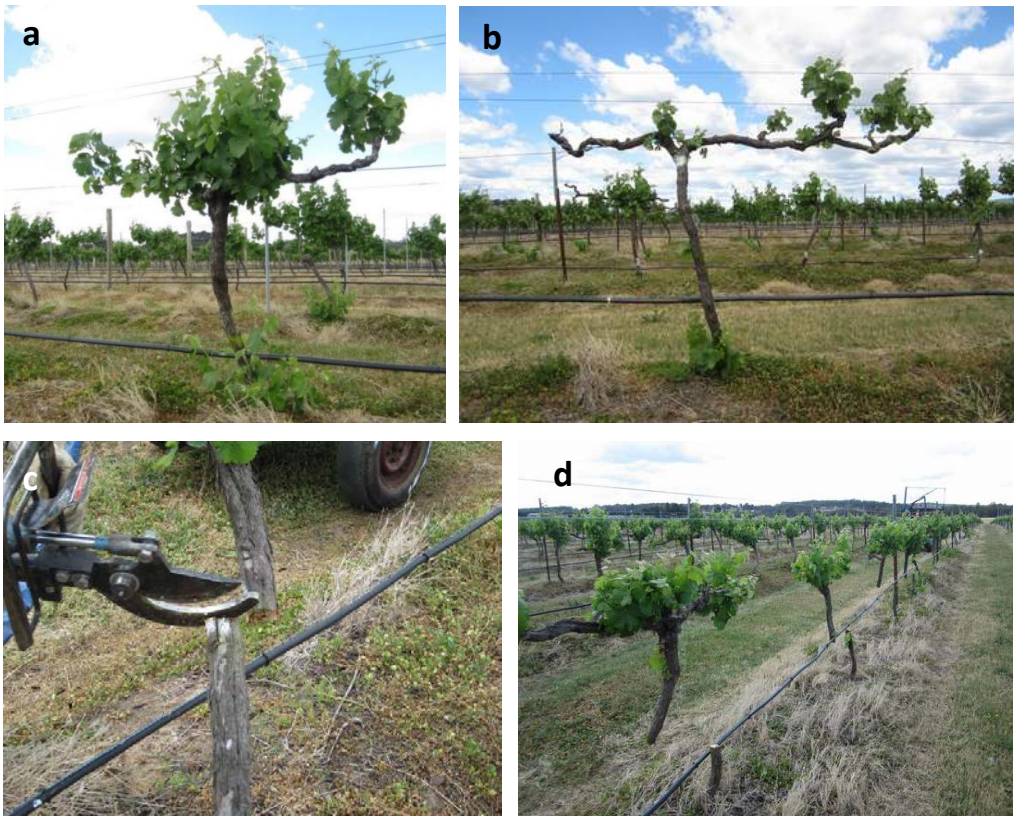


Figure 9.1 (a-b) Severity of dieback in HV1 Hunter Valley, (c) trunk being cut with hydraulic loppers at mid-point between ground and crown and (d) low-cut vines.



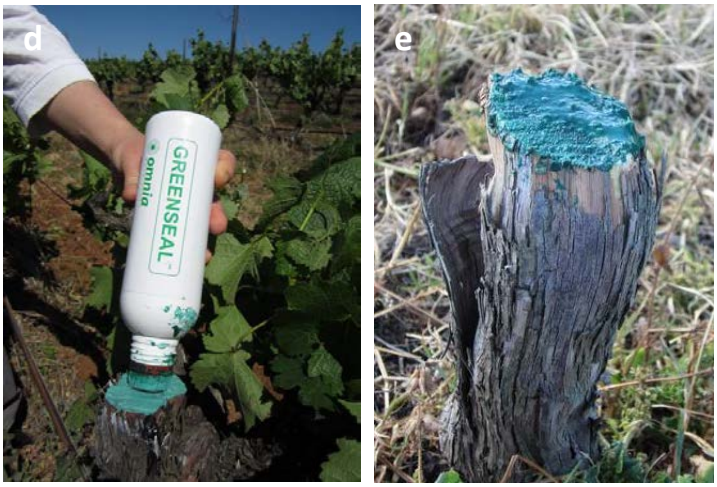


Figure 9.2 (a-c) Trunk cross-sections of symptomatic vines showing necrotic staining in the form of a wedge and/or central staining and (d-e) pruning wound dressing applied following remedial surgery.

9.1 Results

9.2.1 Disease severity pre-remedial surgery

The disease severity at the time of remedial surgery ranged from 74 to 77% for HV1, 53 to 65% for HV2 and 16 to 33% in H1 (Fig. 3). Following remedial surgery, the severity of internal wedge-shaped staining in the cross-section of the trunks at HV1 was 4, 8 and 19% for low, mid and high cut vines, respectively. At HV2, the wedge-shaped staining was 5, 14 and 26%, respectively and at H1, the wedge-shaped staining was 0.6, 2.8 and 0.8%, respectively. The severity of central staining in the cross-section of the trunks ranged from 4 to 19%, 3 to 4% and 2 to 6% for HV1, HV2, and H1, respectively. Botryosphaeriaceae were isolated 100% of symptomatic wood samples from HV1 and Hv2 and 26% from H1. No *Eutypa lata* was isolated from symptomatic samples.

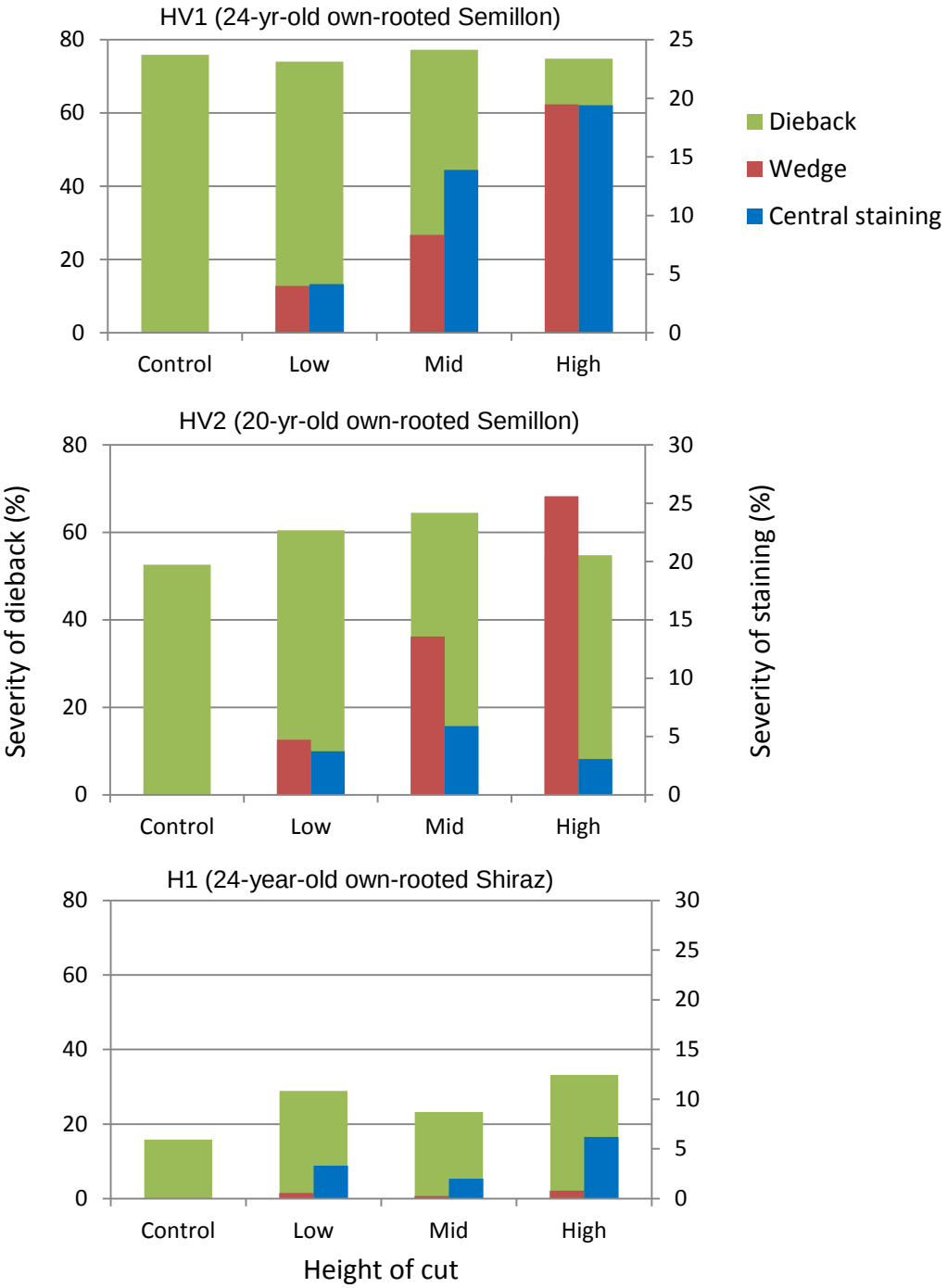


Figure 9.3. The severity of dieback assessed immediately prior to remedial surgery (green columns) and the severity of necrotic staining in the form of a wedge (red) or central (blue) staining around the pith assessed following remedial surgery for three vineyards in the Hunter Valley (HV1 and 2) and at Harden (H1).

9.2.2 Shoot production

The first assessment for water shoot production following remedial surgery was in May 2014. For HV1 grafted vines, 15% of the low cut vines produced water shoots from the scion while 45% of these vines produced water shoots from the rootstock (Figure 9.4; Figure 9.5). For mid cut vines, 50 and 32% of the vines produced water shoots from the scion and rootstock, respectively, while 64 and 14% of the high cut vines produced water shoots from the scion and rootstock, respectively. In October 2014, scion shoot production for mid and high cut vines decreased to 49 and 58%, respectively, while

16% of low-cut vines had produced shoots. The shoot production from rootstocks, however, increased to 48, 37 and 28% for low, mid and high cut vines, respectively. In November 2015, 33% of the vines were lost (dead or missing) due to a severe storm and flooding that occurred in April 2015. Furthermore, 21% of the high cut vines were mistakenly reworked by contractors. For the remaining vines, scion shoot production for low, mid and high cut vines, was 30, 55 and 45%, respectively. The rootstock shoot production was 19-42%. In November 2016, scion water shoot production was 10, 32 and 18% for the surviving low, mid and high cut vines, respectively. The shoot production from rootstocks was 10-18% for all cut vines.

In contrast, for own-rooted vines in HV2 and H1, 97-100% of vines had produced water shoots in each cut height in all assessment periods (Figure 9.4).

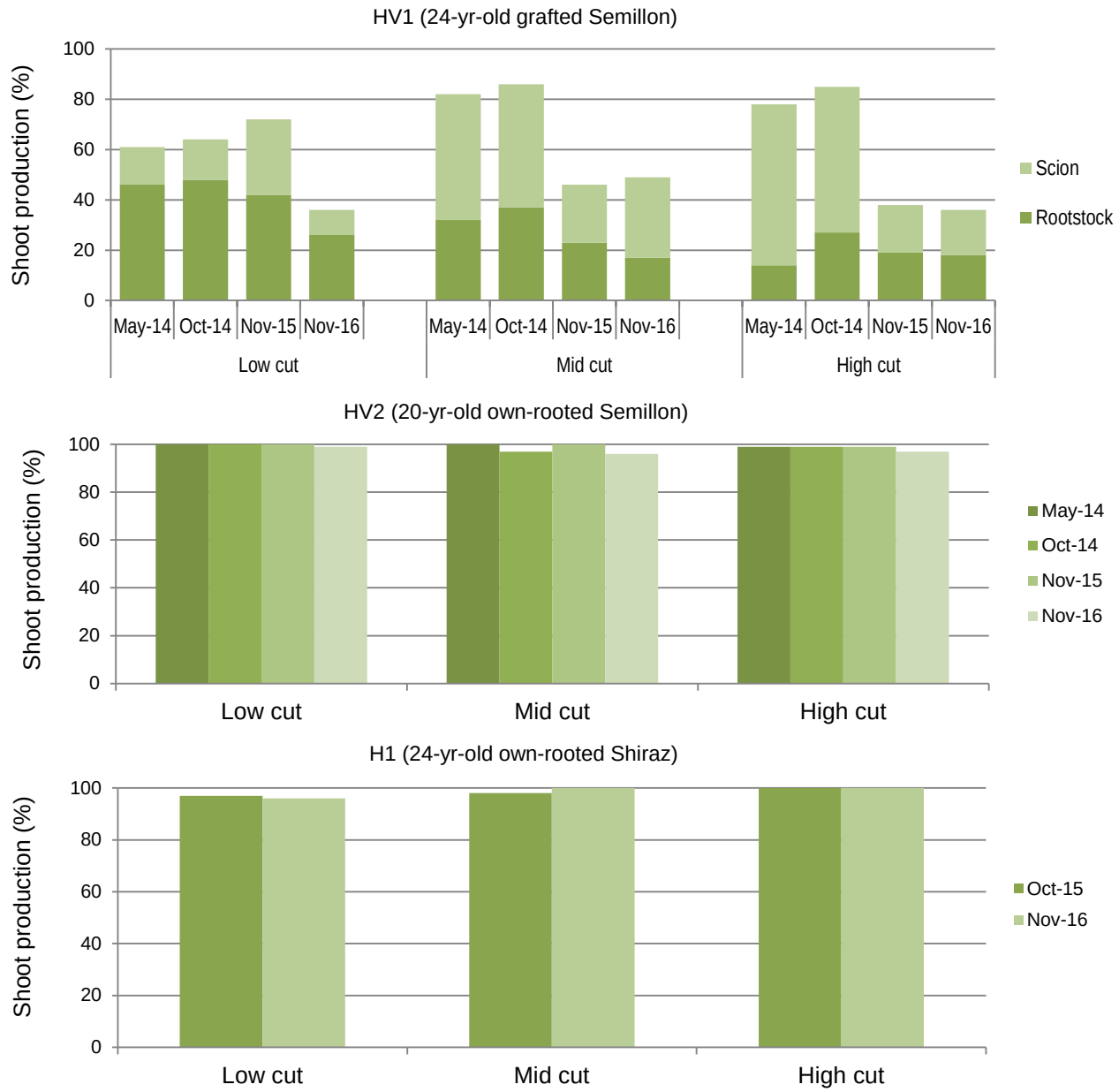


Figure 9.4. Production of water shoots in the growing seasons following remedial surgery for Hunter Valley (HV1 and HV2) and Harden (H1) vineyards.



Figure 9.5. a) Low cut grafted vine with water shoots growing from the rootstock in the Hunter Valley (HV1); b) High cut own-rooted vine with new shoot growing from the base at Harden (H1).

9.2.3 Disease severity post-remedial surgery

For HV1 grafted vines, the initial severity of dieback in the uncut vines (non-treated control) was 76% and increased to 87% by October 2014 (Figure 9.6). However, the following year 93% of the uncut vines were mistakenly reworked by contractors. In November 2015, 97 uncut vines from the adjacent row were tagged and assessed for dieback severity to replace the lost vines. The initial severity of dieback for the replacement vines in 2015 was 83% and increased to 92% by November 2016. No disease symptoms were observed on any treated vines for all four assessment periods.

For HV2 own-rooted vines, the initial severity of dieback in the uncut vines (non-treated control) was 43% and increased to 62% by November 2016 (Figure 9.6). A decrease in severity of dieback was observed at the second assessment (25%; October 2014) was mostly like due to the vigorous vine canopy masking the dieback symptoms. No disease symptoms were observed on any treated vines for all assessment periods.

For H1 own-rooted vines, the mean severity of dieback for uncut vines (non-treated control) was 16% that increased to 19% and 45% following assessment periods (Figure 9.6). No disease symptoms were observed on any treated vines in all assessment periods.

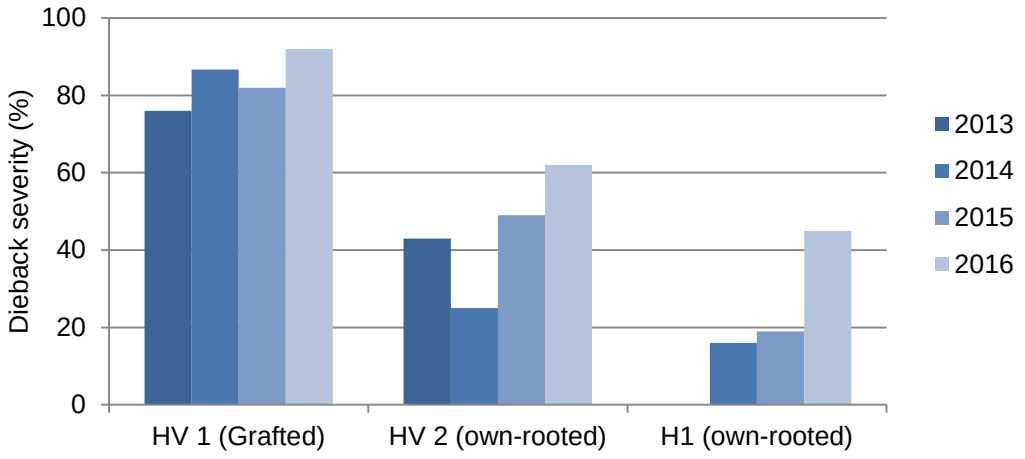


Figure 9.6. Assessment of dieback severity for non-treated control vines (uncut) in three vineyards from 2013 - 2016.

9.3 Discussion

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, the three year data obtained in 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, tended to produce shoots from the rootstock rather than the scion. These results suggest that remedial surgery may be more successful in own-rooted vines than grafted vines. Future research (SAR 1601) will set-up new trials on grafted vines to further elucidate the effect of reworking on these vines. It will also evaluate novel methods of water shoot induction to improve success of the technique.

In this study, the severity of dieback in untreated vines increased by 16-29% after three years of monitoring. This indicated that if infected vines were not reworked, the dieback severity would have increased by 5-10% each year, eventually leading to the death of vines if no interventions are applied. In HV1, more than half of the treated vines were lost due to flooding while the average severity of dieback in the untreated vines had already reached 92%. Thus, further monitoring of these vines in the new project will not continue. However, the trials in HV1 and H2 have demonstrated the short-term success of remedial surgery for control of BD in own-rooted vines. Thus, monitoring of disease recurrence for these vines will continue for at least three more growing seasons as part of project SAR 1601.

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

10 DISEASE SUSCEPTIBILITY

10.1 Introduction

There have been limited reports of resistance or tolerance of *Vitis vinifera* cultivars to trunk disease. Carter (1991) cited a report by Dubos (1987) on the resistance or susceptibility to eutypa dieback (ED) of cultivars typically grown in France based on foliar symptoms in the vineyard. Of 32 cultivars assessed, five were categorised as resistant (cvs. Aligote, Grolleau, Merlot, Semillon and Sylvaner) and all others listed as moderately to highly susceptible. Based on three surveys conducted in South Australia over the past 40 years (Wicks 1975, Highet and Wicks 1998, Loschiavo *et al.* 2007), the Grenache, Cabernet Sauvignon and Shiraz cultivars were recorded to have the highest incidence of eutypa dieback foliar symptoms and Merlot, Riesling, Pinot Noir, Sauvignon Blanc, Chardonnay and Semillon cultivars the least. The growth of *E. lata* in grapevine wood also varies and Merlot, Gamay, Grenache and Semillon cultivars were recorded to have half of the rate of dieback of Cabernet Sauvignon and Shiraz (Sosnowski *et al*. 2007b).

For botryosphaeria dieback (BD), studies on lesion length in canes of several cultivars of *V. vinifera* and other *Vitis* spp. indicated variation in susceptibility (Savocchia *et al.*, 2007; Pitt *et al.*, 2013; Travadon *et al.* 2013, Billones-Baaijens *et al.* 2014; Qiu 2014).

The SARDI germplasm collection in the Barossa Valley consists of a broad range of *V. vinifera* cultivars and clones sourced from around the world. This study utilised the collection and included visual assessment of symptoms of trunk disease severity in cultivars and clones and evaluated the rate of pathogen colonisation in detached canes, potted and vineyard vines. It aimed to identify resistant or tolerant cultivars and clones, in order to provide recommendations for future plantings to reduce susceptibility to trunk diseases. The CSIRO is breeding rootstocks with improved qualities, including tolerance to foliar diseases, and this study took the opportunity to screen genotypes in the program with the aim of providing selections for plant breeders that may reduce susceptibility to ED and BD pathogens.

10.2 Methods

10.2.1 Cultivar susceptibility

Field vine symptom assessment
The SARDI germplasm cultivar collection, located at the Nuriootpa Research Centre, South Australia, consists of 83 red and 95 white wine grape cultivars (*V. vinifera*) sourced from around the world. Vines were planted between 1977 and 1982 with one panel (three or four vines) per cultivar. All vines have been cordon trained and spur pruned, with no specific strategies to control trunk disease.

On 7 and 15 November in 2012 and 2013, respectively, vines were visually assessed for (i) severity of eutypa dieback foliar symptoms and (ii) overall severity of trunk disease symptoms (eutypa and botryosphaeria dieback), which, in addition to foliar symptoms, included the presence of dead spurs, cordon dieback and trunk cankers (Figure 10.1). Ratings were on a percentage scale (0% - non-symptomatic, 100% - dead) and were averaged for each cultivar.

Detached cane assay assessment
Detached cane assays (DCA, Ayres *et al*. 2011) were established on 20 December 2013 and 13 July 2016 for *E. lata*, and 8 January 2014 and 11 December 2015 for *D. seriata*, in repeated experiments to evaluate cultivar susceptibility to infection. Canes were cut into 10 cm single-node sections with the top surface 1 cm above the node and placed into holes 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. Boards were set up to hold 18 *V. vinifera* cultivars selected to represent the least symptomatic, according to the visual symptom assessments, and compared with the susceptible cultivars Shiraz and Sauvignon Blanc. With four canes per treatment placed in columns on the boards, two treatments per column, the experiments were established as randomised block designs with five replications (boards). The boards with canes were floated on tap water in plastic tubs in a greenhouse maintained at approximately 25°C.

Wounds on top of canes were moistened by spraying with SDW immediately prior to inoculation with approximately 500 spores of *E. lata* or 1000 spores of *D. seriata* suspended in 20 µL SDW and applied using a pipette. One tray was set up as a non-inoculated control.

The experiments were harvested for assessment four weeks after establishment. Bark was removed from each cane using a sharp knife, cane diameter measured midway between two nodes and the exposed wood was surface sterilised in 2.5% sodium hypochlorite (NaOCl) for 10 minutes before being washed twice in SDW. Starting immediately below the inoculation point, cuts were made using sterile secateurs every 2 mm along the canes for a distance of 30 mm. Segments were placed sequentially on PDA amended with streptomycin sulfate (100 mg/L) and incubated for a week with 12 hr day/night light cycle at 25°C. Each wood segment was then assessed for the presence or absence of outgrowth of *E. lata* or *D. seriata*, and the distance the fungus extended from the inoculation point was recorded. Data were subjected to analysis of variance and least significant difference (LSD) at the 5% level was used for all pairwise comparisons; standard error of the means was calculated. Correlation between pathogen recovery distance and cane width was determined using regression analysis.



Figure 10.1. Rating scales used for visual assessment of eutypa dieback foliar symptoms and overall grapevine trunk disease.

Potted vine assessment
In spring 2014, canes (approx. 30 cm long) of each cultivar used in the DCA were propagated by first placing them upright into trays filled with a vermiculite/perlite mix 10 cm deep, placed in a greenhouse maintained at approx. 25°C, and watered twice weekly (Figure 10.2a). Canes were callused and, once roots had formed (approx. eight weeks), they were transferred to 15 cm diameter pots filled with University of California (UC) potting mix (Baker *et al.* 1957) and placed on a bench in the shadehouse (Figure 10.2b).

In February 2015, stems were cut back to expose a pruning wound approx. 1cm above a live node. Mycelial broths of *E. lata* (isolate DAR82124) and *D. seriata* (isolate DAR79140) were made based on the method of Travadon *et al.* (2013). Briefly, plugs from cultures grown on PDA were inoculated into Erlenmeyer flasks containing potato dextrose broth (PDB), placed on an orbital shaker at 150 rpm and grown at 25°C for 3 and 5 days for *D. seriata* and *E. lata,* respectively. Using a hand-held disperser, the liquid cultures were homogenised and 2 mL of this homogenised starter culture was inoculated into Erlenmeyer flasks containing PDB. After a further 3 and 5 days, the liquid cultures were homogenised and the concentration of mycelial fragments which were <0.5 mm in length was estimated with a haemocytometer. The final concentration of inoculum was adjusted with sterile water to 1 x 10^6 fragments/mL. Using a pipette, 20 µL of the mycelial broth was placed onto the exposed pruning wounds which had been moistened by spraying with SDW (Figure 10.2c). In order to keep the wounds moist, plastic bags (100 x 150 mm) which had been moistened by spraying with SDW were placed over the wounds and secured with snap locks (Figure 10.2d). Non-inoculated and broth-only (without mycelium) controls were included for comparison. After three days, the bags were removed. Vines were monitored for foliar symptoms during spring 2015.



Figure 10.2. Potted vine assay: a) propagation of canes in vermiculite/perlite on the bench in greenhouse, b) potted vines on bench in shadehouse, c) inoculating stem wounds with mycelial broth using a pipette and d) moistened plastic bags over wounds.

To determine the extent to which *E. lata* and *D. seriata* colonised the stems, vines were removed from pots in April 2016, and shoots and roots were excised before placing in cool storage. For assessment, bark was removed from stems using a sharp knife, cane diameter measured midway between two nodes and recorded and the extent of staining 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 300 mm below the inoculated wound and transferred to agar. 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. Data were subjected to analysis of variance and least significant difference (LSD) at the 5% level was used for all pairwise comparisons; standard error of the means was calculated. Correlation between pathogen recovery distance and both extent of staining and stem diameter was determined using regression analysis.

Field vine inoculation assessment
In the germplasm collection at the Nuriootpa Research Centre, one-year-old canes of the 20 cultivars evaluated in the DCA and potted vine experiments were pruned, using secateurs, to at least 20 cm length on 9 July in 2014 and 8 July 2015. Wounds were moistened by spraying with SDW immediately prior to inoculation with approximately 500 spores of *E. lata* or 1000 spores of *D. seriata* suspended in 20 µL SDW and applied using a pipette. Ten canes per vine (three vines per cultivar) were inoculated with each pathogen.

To determine the extent of colonisation by *E. lata* and *D. seriata*, canes were removed from vines on 8 July 2015 and 28 July 2016 and placed in cool storage. Assessment was as described above for potted vines except that samples were taken up to 200 mm below the wound. A statistical analysis of data provided means and standard errors for each pathogen and cultivar combination. Correlations between pathogen recovery distance and both cane width (measurements from DCA) and extent of staining was determined using regression analysis.

10.2.2 Clonal susceptibility

Symptom assessment in the field
Vines of the cultivars Cabernet Sauvignon, Shiraz and Chardonnay in the SARDI clonal collection, located at the Nuriootpa Research Centre were used. These vines had been planted between 1969 and 1996, one panel of three vines per clone, and cordon trained and spur pruned, with no specific strategies to control trunk diseases. On 28 October 2014, vines were visually assessed as described in Section 10.2.1

10.2.3 Rootstock susceptibility

Detached cane assay assessment
Detached cane assays (Ayres *et al*. 2011) were established on 5 March 2014 and 17 March 2016 to evaluate susceptibility to both eutypa and botryosphaeria dieback by cutting canes into 10 cm single-node sections and placing into polystyrene boards as described in section 10.2.1. Boards were designed to hold 19 rootstock genotypes from *V. vinifera* (Riesling) *x V. cinerea* lines (2014) and 17 genotypes with a range of parentage (2016), and Shiraz for comparison, with four canes per treatment placed in columns on the boards, two treatments per column. The experiments were established as randomised block designs with five replications (boards). The boards with canes were floated on tap water in plastic tubs in a greenhouse maintained at approximately 25°C. Inoculation and assessment were conducted using the methods described in section 10.2.1.

10.3 Results

10.3.1 Cultivar susceptibility

10.3.1.1 Field vine symptom assessment

The severity of overall trunk disease symptoms (eutypa and botryosphaeria dieback) and foliar symptoms of eutypa dieback varied substantially amongst the cultivars (Figure 10.3 and Figure 10.4). In general, the mean severity of foliar symptoms of eutypa dieback was similar for red and white wine

cultivars (5.6 and 4.1%, respectively). The severity of overall trunk disease symptoms was greater in red (41%) than white (30%) wine cultivars.

Foliar symptoms of eutypa dieback were apparent on 33 of the 83 red wine cultivars, severity ranging from 1-39% (Figure 10.3), and on 47 of the 95 white wine cultivars with 1-31% severity (Figure 10.4). In terms of overall trunk disease symptoms, all 86 red and 91 white cultivars exhibited severity from 1-98%. Correlations between foliar and overall symptoms were similar for white (R^2 = 0.37) and red (R^2 = 0.28) cultivars. No symptoms were observed on four white wine cultivars but all red wine cultivars had symptoms. Furthermore, 15 red and 26 white cultivars exhibited less than 10% mean severity of trunk disease symptoms. The mean severity of trunk disease symptoms was greater than 80% for 13 red and five white wine cultivars.

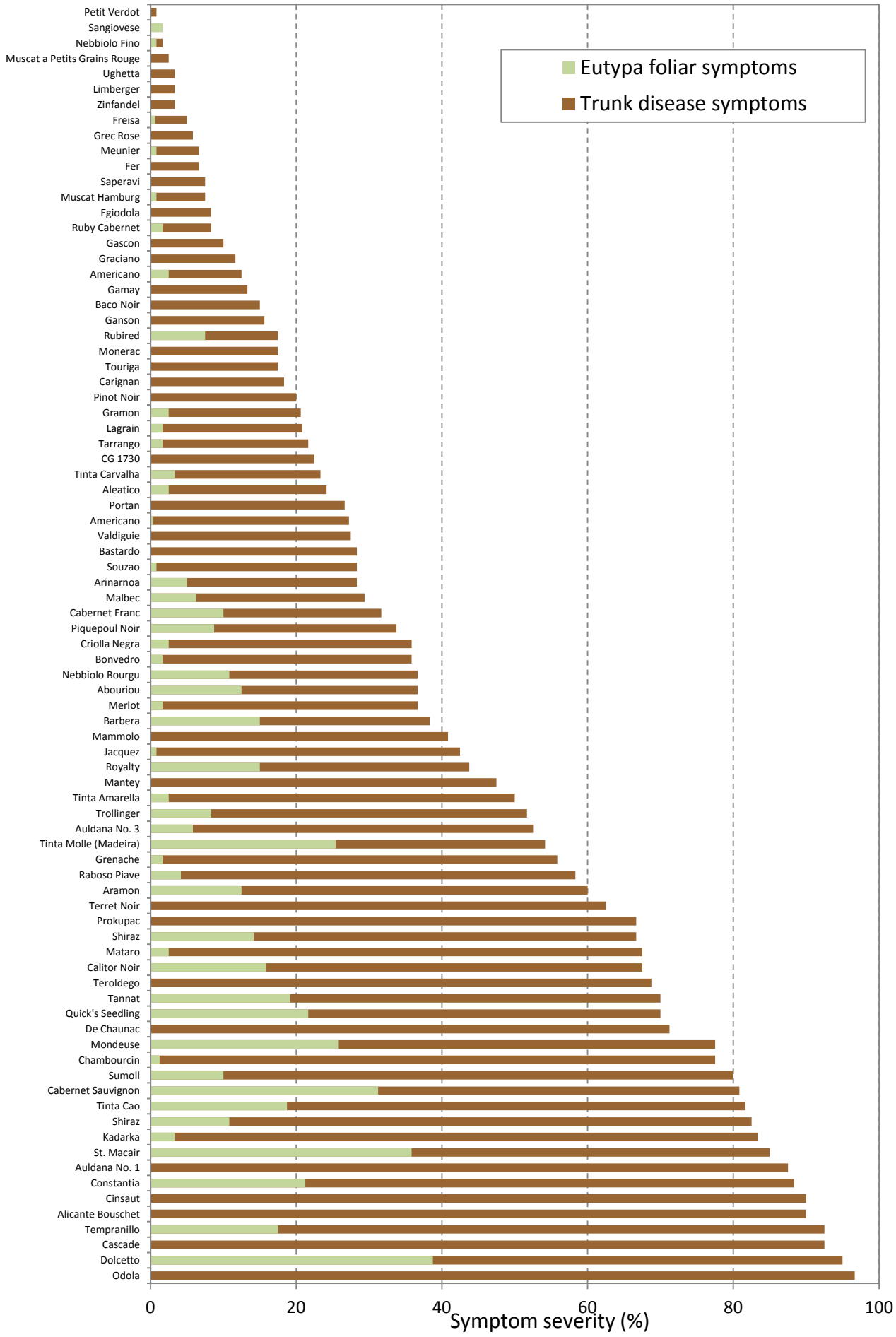


Figure 10.3 Severity of overall trunk disease symptoms of eutypa and botryosphaeria dieback (brown) and foliar symptoms of eutypa dieback (green) on red wine cultivars (*Vitis vinifera*) in the germplasm collection, based on the mean of assessments made in November 2012 and 2013 at the Nuriootpa Research Centre, Barossa Valley, South Australia.

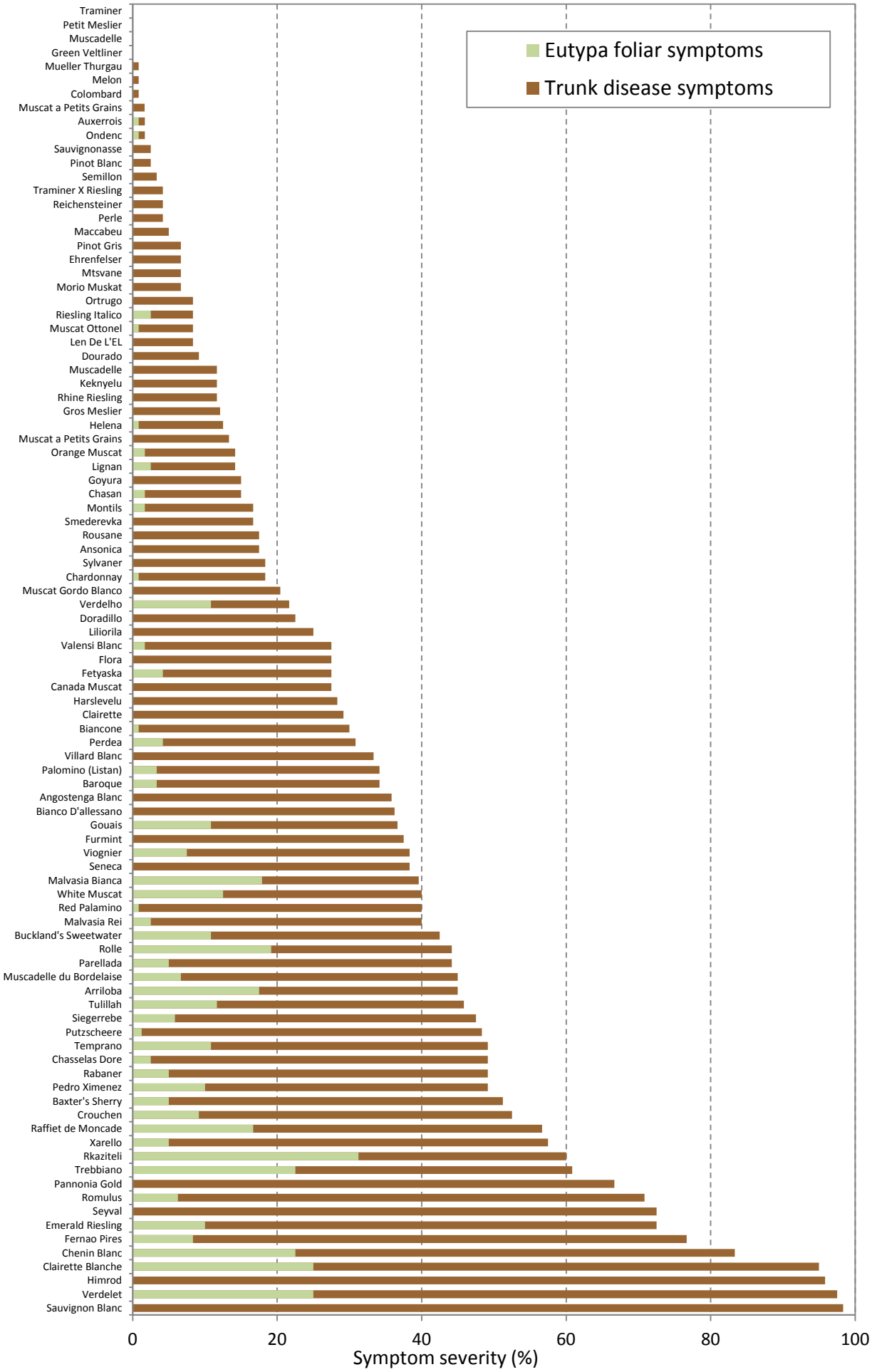


Figure 10.4 Severity of overall trunk disease symptoms of eutypa and botryosphaeria dieback (brown) and foliar symptoms of eutypa dieback (green) on white wine cultivars (*Vitis vinifera*) in the germplasm collection, based on the mean of assessments made in November 2012 and 2013 at the Nuriootpa Research Centre, Barossa Valley, South Australia.

10.3.1.2 Detached cane assay assessment

In the eutypa dieback DCA, *E. lata* colonised canes of all cultivars between 4 and 23 mm from the wound site over the four week incubation period in both experiments (Figure 10.5). *E. lata* was not recovered from non-inoculated canes. In the susceptible cultivars, Shiraz and Sauvignon Blanc, *E. lata* was recovered 20-23 mm from the wound site. Of the 18 cultivars which expressed the least symptoms in the field assessments, 13 and nine were colonised to significantly less mean distance than the susceptible cultivars in Experiments 1 and 2, respectively. Canes of the Petit Meslier, Petit Verdot, Auxerrois, Ughetta, Muscat a Petit Grains Rouge, Nebbiolo Fino and Muscadelle cultivars were colonised significantly less than the susceptible cultivars in both experiments, *E. lata* being recovered 4-14 mm from the wound.

In the botryosphaeria dieback DCA, *D. seriata* colonised canes of all cultivars from 7-27 mm from the wound site over the four-week incubation period in both experiments (Figure 10.6). *D. seriata* was not recovered from non-inoculated canes. In canes of the susceptible cultivars, Shiraz and Sauvignon Blanc, *D. seriata* was recovered 20-21 mm and 12-14 mm from the wound site in Experiments 1 and 2, respectively. Of the 18 cultivars from the germplasm collection, five and seven were colonised to significantly less mean distance than the susceptible cultivars in Experiments 1 and 2, respectively. Canes of the Green Veltliner and Muscadelle cultivars were colonised significantly less than the susceptible cultivars in both experiments, *D. seriata* being recovered less than 17 and 8 mm from the wound in Experiments 1 and 2, respectively.

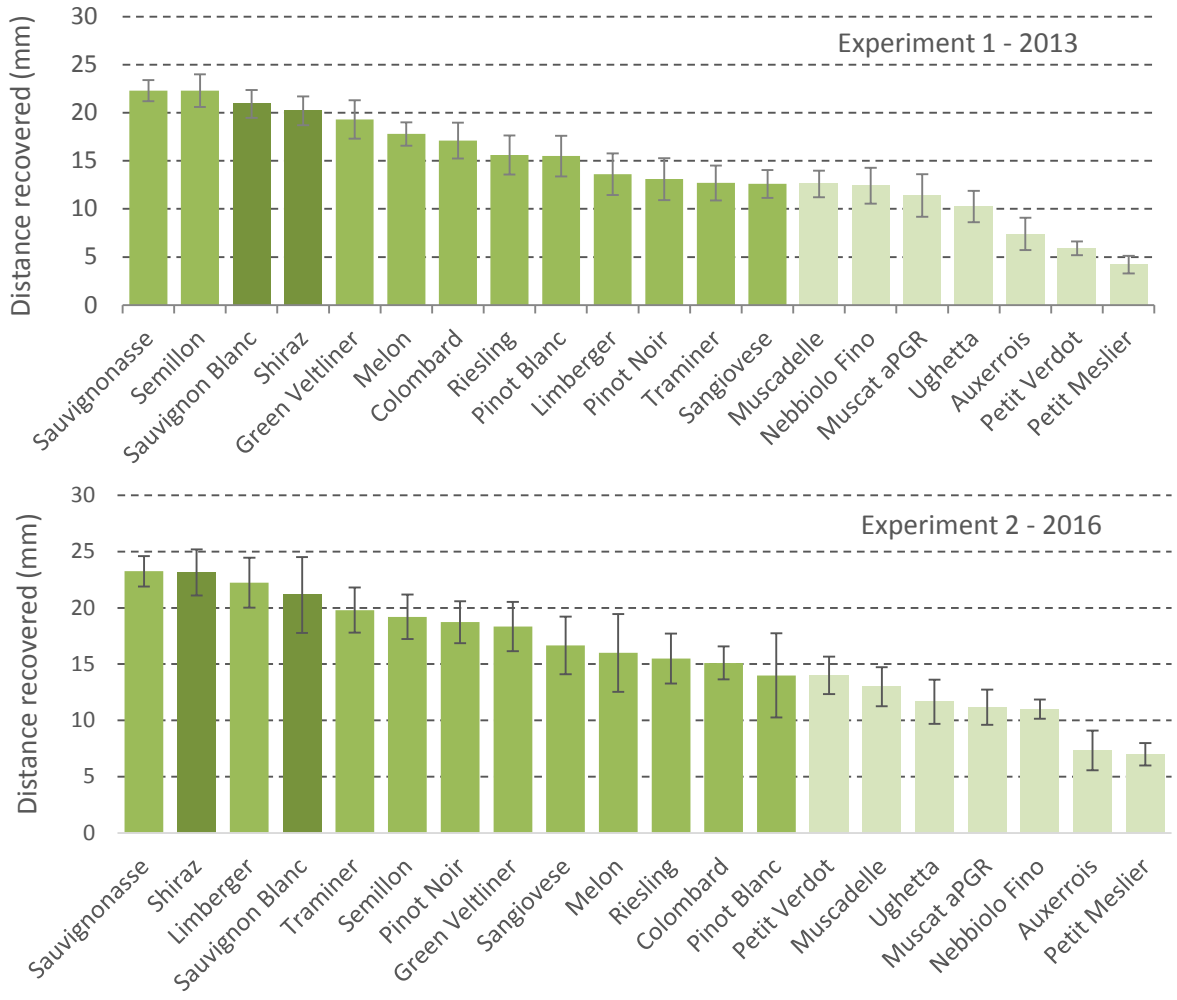


Figure 10.5 Recovery distance of *Eutypa lata* (eutypa dieback), four weeks after applying spores to the cut surface of canes detached from selected cultivars in the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia in a repeated experiment. Bars represent standard error of the mean. Darkest shading – representative susceptible cultivars, lightest shading – cultivars consistently colonised least in both experiments. Muscat aPGR = Muscat a Petit Grains Rouge.

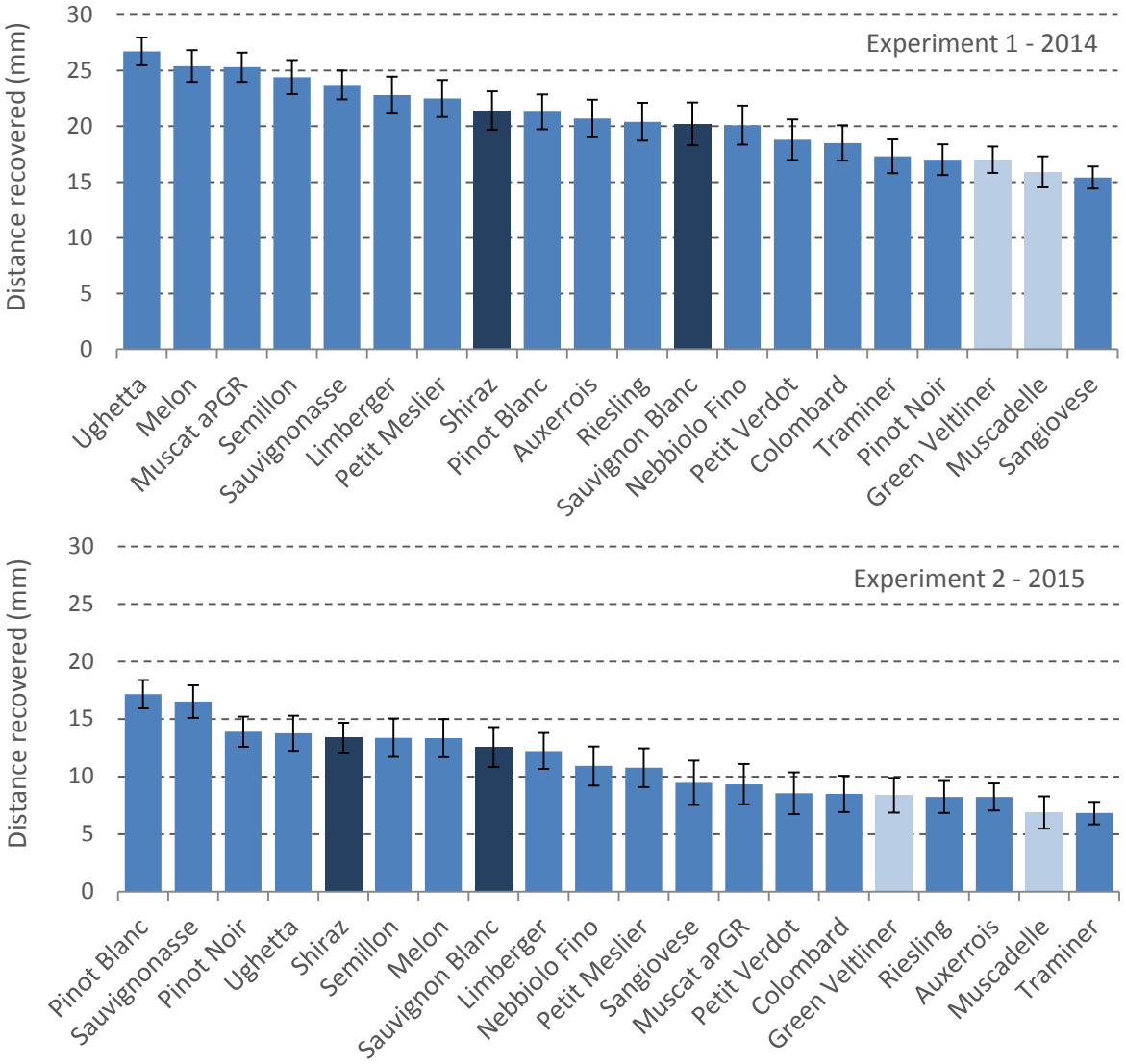


Figure 10.6 Recovery distance of *Diplodia seriata* (botryosphaeria dieback), four weeks after applying spores to the cut surface of canes detached from selected cultivars in the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia in a repeated experiment. Bars represent standard error of the mean. Darkest shading – representative susceptible cultivars, lightest shading – cultivars consistently colonised least in both experiments. Muscat aPGR = Muscat a Petit Grains Rouge.

The mean cane width of cultivars from all experiments combined varied from 3.9 to 6.0 mm (Figure 10.7). The susceptible cultivars Shiraz and Sauvignon Blanc had mean diameters of 5.9 and 5.7 mm, respectively. Cultivars with the smallest mean diameter included: Petit Meslier, Petit Verdot, Muscadelle, Ughetta, Melon and Auxerrois, measuring 3.9 to 4.8 mm, significantly less than most other cultivars.

Regression analysis was conducted to compare distance of pathogen recovery with cane diameter, and for eutypa dieback there was a positive linear trend in that *E. lata* was recovered the furthest distance in the canes with the greatest diameter, although R^2 values were only 0.52 and 0.35 for Experiments 1 and 2, respectively (Figure 10.8a-b). However, there was no such relationship for botryosphaeria dieback (Figure 10.8c-d).

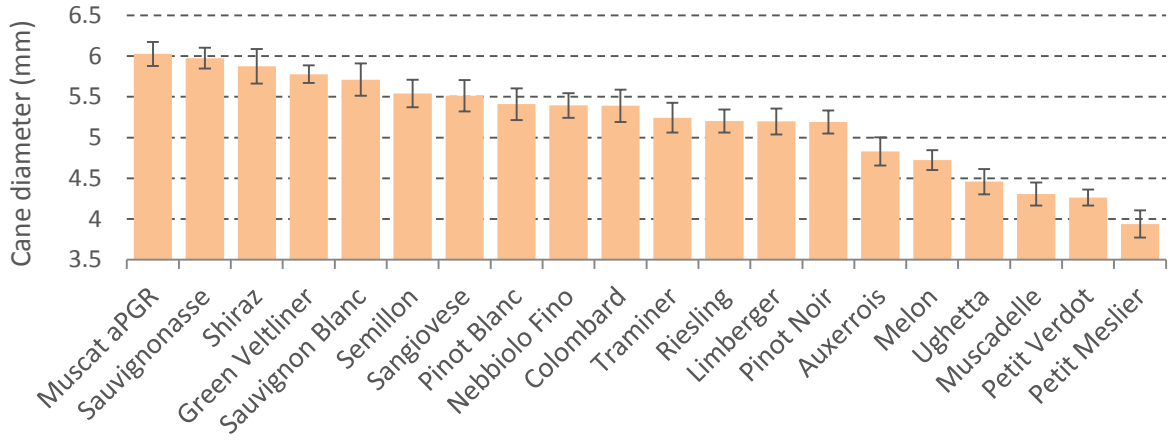


Figure 10.7 Diameter of debarked canes detached from selected cultivars in the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia (data from all experiments combined). Bars represent standard error of the mean. Muscat aPGR = Muscat a Petit Grains Rouge.

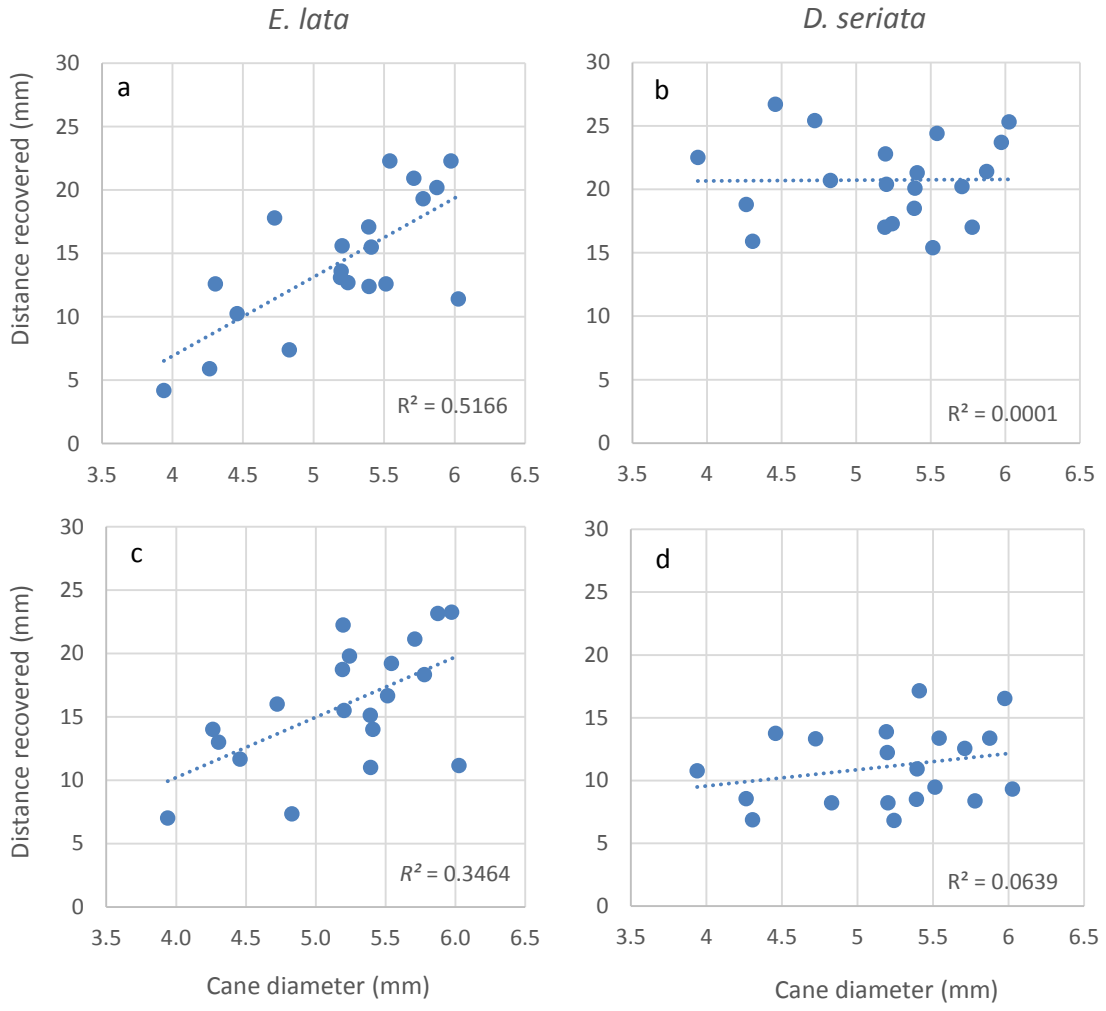


Figure 10.8 Relationship between mean distance *Eutypa lata* (a, c) and *Diplodia seriata* (b, d) were recovered from detached canes and the diameter of detached canes of cultivars taken from the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia, in Experiment 1 (a-b) and Experiment 2 (c-d).

10.3.1.3 Potted vine assessment

No foliar symptoms were observed in spring 2015.

For the eutypa dieback potted vine experiment, isolations from stems did not yield sufficient incidence of *E. lata* to permit robust statistical analysis of data on susceptibility of stems to colonisation. Although isolation plates were sometimes contaminated with other microorganisms, many stem segments that were not overgrown with contaminants did not yield *E. lata,* indicating that the fungus had not colonised the wounds.

For the botryosphaeria dieback potted vine experiment, *D. seriata* colonised stems of all cultivars mean distances of between 20 and 73 mm from the wound site (Figure 10.9). In canes of the susceptible cultivars Shiraz and Sauvignon Blanc, *D. seriata* was recovered a mean distance of 56 and 30 mm from the wound site, respectively. Of the 18 cultivars selected from the germplasm collection, stems of four (Ughetta, Petit Verdot, Colombard and Nebbiolo Fino) were colonised less than Shiraz, *D. seriata* being recovered less than 32 mm from the wound. However, no cultivar was colonised less than Sauvignon Blanc. Mean distance at which staining was recorded on stems ranged between 15 and 74 mm (Figure 10.9). On average, *D. seriata* was recovered 5-38 mm ahead of staining in 14 cultivars and 1-4 mm behind staining in six cultivars. Mean diameter of stems ranged between 3 and 7 mm, and those for Shiraz and Sauvignon Blanc were 6.3 and 5.4 mm, respectively.

The greatest distance *D. seriata* was recovered from a single cane was 170 mm (cv. Limberger; Table 10.1). *D. seriata* was recovered from non-inoculated canes of some cultivars (cv. Green Veltliner 140 mm from wound site, Muscat a Petit Grains Rouge 150 mm, Pinot Noir 20 mm, Sauvignonasse 190 mm and Sauvignon Blanc 10 mm). *D. seriata* was not isolated from canes inoculated with broth-only.

Mean recovery distance of *D. seriata* showed a weak correlation with mean staining distance (R^2 =0.5) and less so with stem diameter (R^2 = 0.2; Figure 10.10)

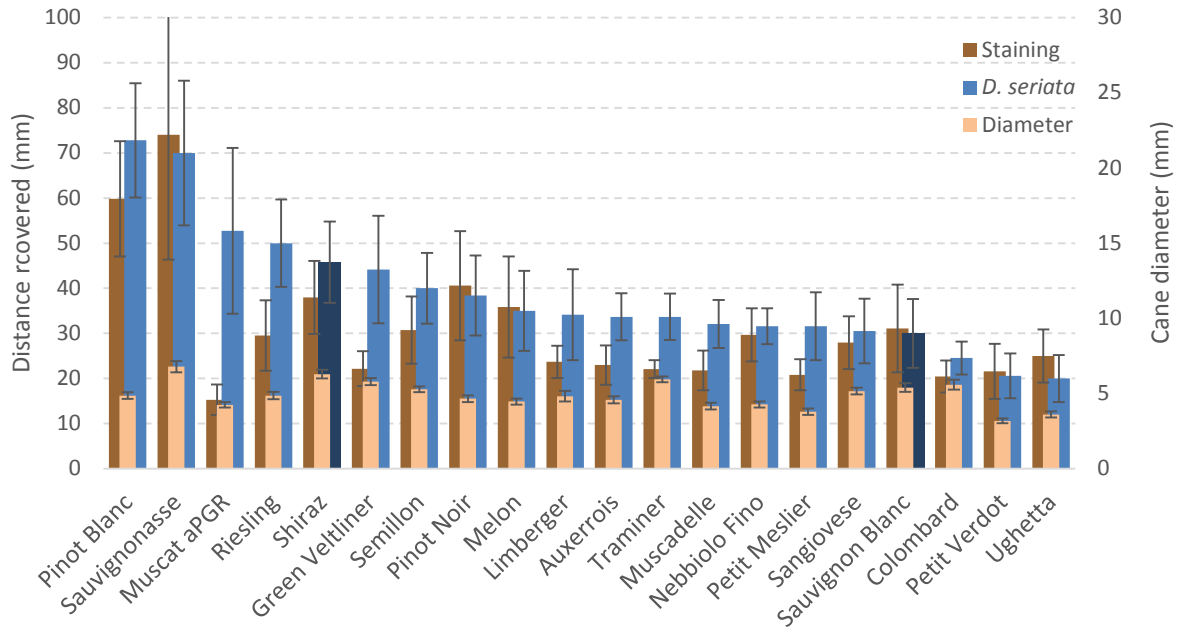


Figure 10.9 Recovery distance of *Diplodia seriata* (blue bars), staining distance (brown bars) and stem diameter (orange bars), 12 months after applying mycelial broth to the cut surface of stems of potted grapevines propagated from selected cultivars in the germplasm collection. Bars represent standard error of the mean. Muscat aPGR = Muscat a Petit Grains Rouge

Table 10.1. Maximum distance (mm) at which *Diplodia seriata* was recovered and staining was recorded below wounds inoculated with mycelial broth for a single stem for each cultivar, along with recovery and staining distance for non-inoculated and broth-only controls.

	Maximum		Non-inoculated		Broth only	
	D. seriata	Staining	*D. seriata*	Staining	*D. seriata*	Staining
Auxerrois	80	80	0	15	0	9
Colombard	50	53	0	25	0	29
Green Veltliner	160	73	140	138	0	12
Limberger	170	60	0	11	0	15
Muscat aPGR	60	36	150	60	0	55
Melon	120	182	0	8	0	10
Muscadelle	80	85	0	11	0	10
Nebbiolo Fino	50	96	0	9	0	15
Pinot Blanc	160	170	0	85	0	n/a
Petit Meslier	120	65	0	16	0	14
Pinot Noir	140	210	20	18	0	14
Petit Verdot	80	115	0	73	0	45
Riesling	110	108	0	11	0	33
Sauvignonasse	130	210	190	145	0	90
Sangiovese	130	104	0	18	0	15
Sauvignon Blanc	130	160	10	8	0	8
Semillon	130	116	0	16	0	10
Shiraz	140	130	0	13	0	14
Traminer	90	99	0	18	0	15
Ughetta	60	90	0	16	0	18

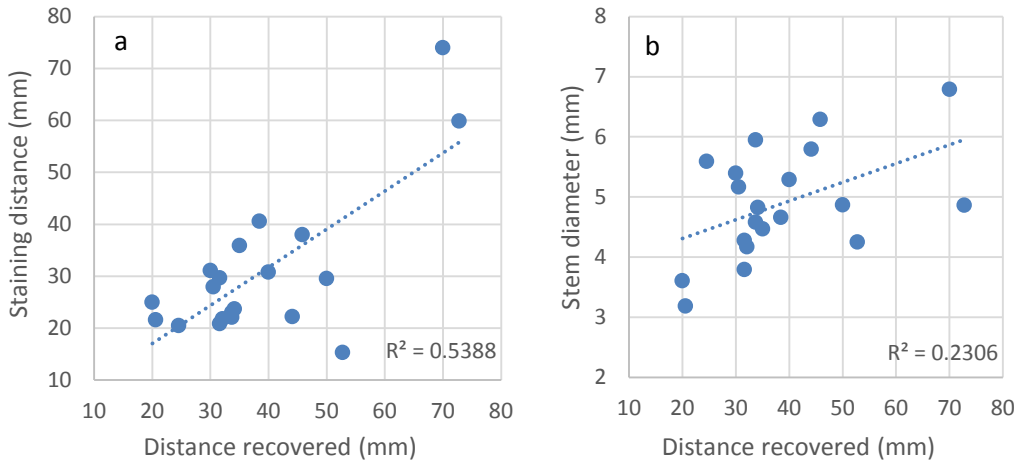


Figure 10.10 Relationship between mean distance *Diplodia seriata* recovered and the (a) staining distance or (b) diameter of stems of cultivars propagated from the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia.

10.3.1.4 Field vine inoculation

Eutypa lata colonised canes of all cultivars to 22-160 mm (2014-15) and 9-139 mm (2015-16) below the wound site (Figure 10.11). In the susceptible cultivars Shiraz and Sauvignon Blanc, *E. lata* was recovered 84 and 80 mm from the wound site, respectively in 2014-15, and 91 and 103 mm, respectively in 2015-16. Of the 18 cultivars which expressed the least symptoms in the field assessments, four and three were colonised to significantly less mean distance than the susceptible cultivars in 2014-15 and 2015-16, respectively. Canes of the Petit Verdot, Muscat a Petit Grains Rouge and Muscadelle cultivars were colonised less than the susceptible cultivars in both trials, with *E. lata* recovered 22-34 mm and 29-59 mm from the wound in 2014-15 and 2015-16, respectively.

Diplodia seriata colonised canes of all cultivars from 4-30 mm (2014-15) and 3-61 mm (2015-16) from the wound site (Figure 10.12). In canes of the susceptible cultivars Shiraz and Sauvignon Blanc, *D. seriata* was recovered 20 and 21 mm from the wound site, respectively in 2014-15 and 42 and 61 mm, respectively in 2015-16. Of the 18 cultivars from the germplasm collection, seven and 17 were colonised to significantly less mean distance than the susceptible cultivars in 2014-15 and 2015-16, respectively. Canes of the Petit Meslier, Melon, Petit Verdot, Ughetta, Muscadelle and Green Veltliner were colonised significantly less than the susceptible cultivars in both trials, *D. seriata* being recovered 4-9 mm and 3-17 mm from the wound in 2014-15 and 2015-16, respectively.

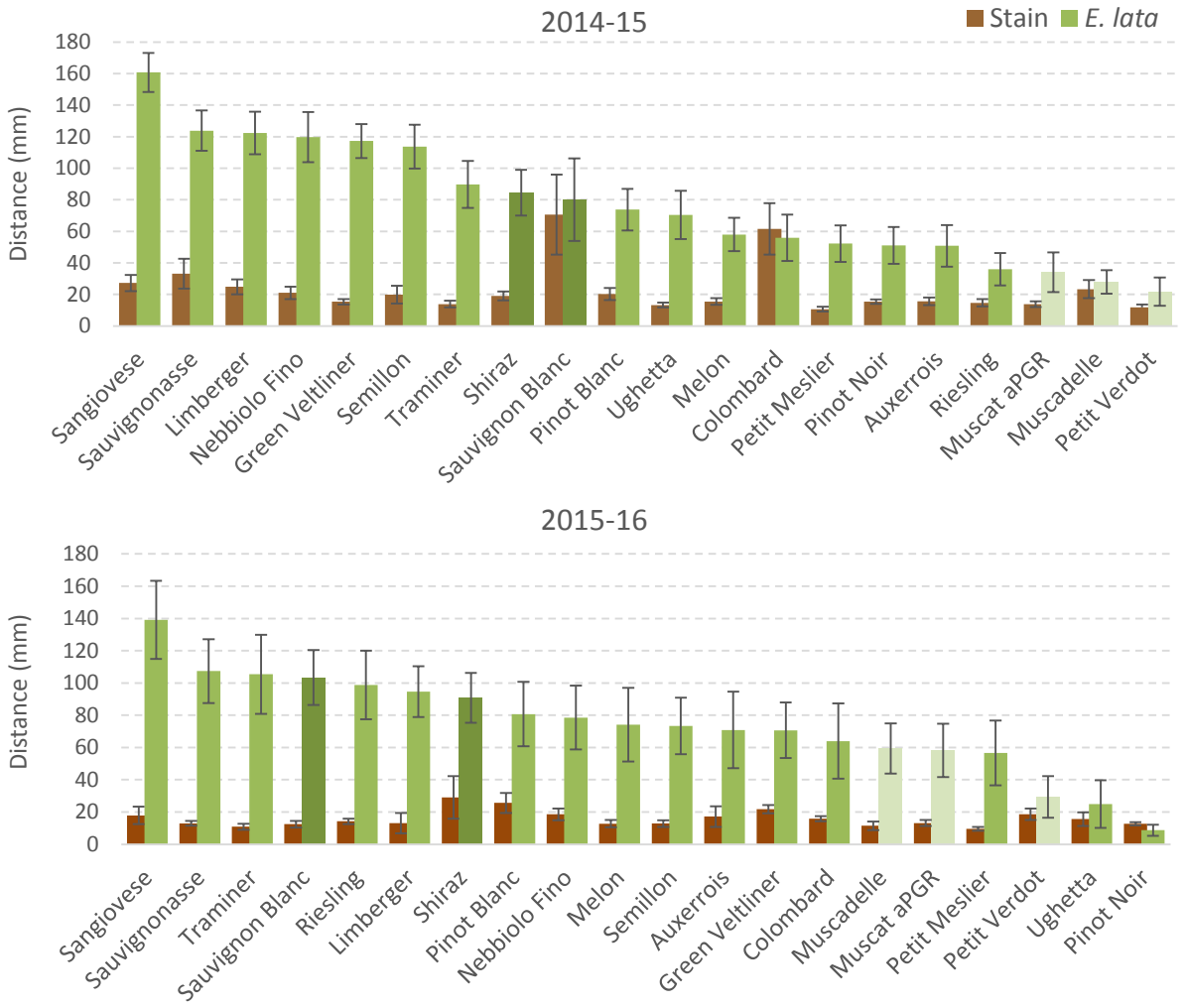


Figure 10.11 Recovery distance (green bars) of *Eutypa lata* (eutypa dieback) and associated staining (brown bars) 12 months after applying spores to the cut surface of attached canes on selected cultivars in the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia. Bars represent standard error of the mean. Darkest shading – representative susceptible cultivars, lightest shading – cultivars consistently colonised least in both experiments. Muscat aPGR = Muscat a Petit Grains Rouge.

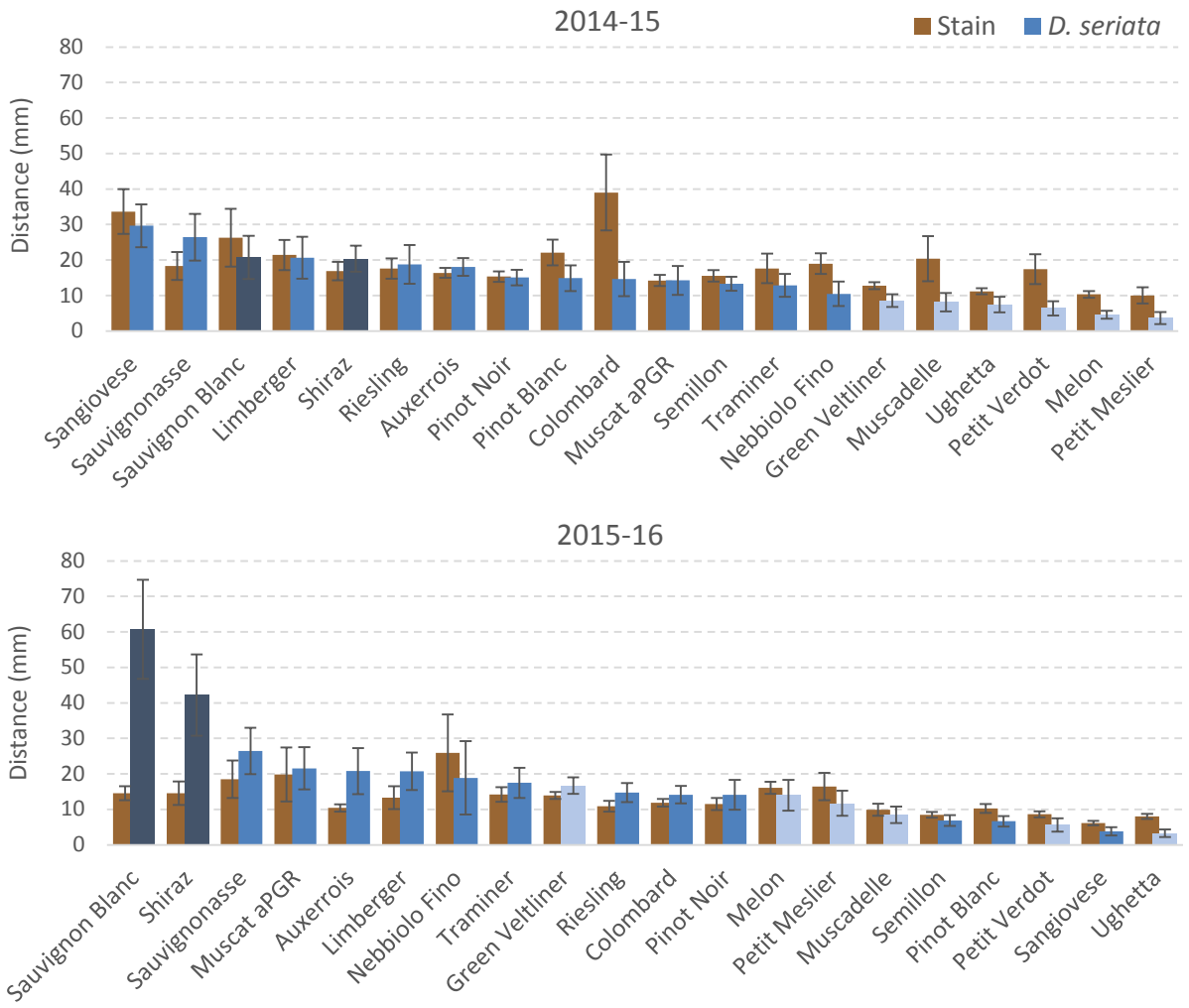


Figure 10.12 Recovery distance (blue bars) of *Diplodia seriata* (botryosphaeria dieback) and associated staining (brown bars) 12 months after applying spores to the cut surface of attached canes on selected cultivars in the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia. Bars represent standard error of the mean. Darkest shading – representative susceptible cultivars, lightest shading – cultivars consistently colonised least in both experiments. Muscat aPGR = Muscat a Petit Grains Rouge.

When considering both pathogens, canes of Petit Verdot and Muscadelle were colonised to a significantly shorter distance from the wound site than the susceptible Shiraz and Sauvignon Blanc cultivars in all four field vine inoculation experiments. Furthermore, canes of Muscat a Petit Grains Rouge, Ughetta, Pinot Noir and Riesling were colonised to a significantly shorter distance from the wound site than the susceptible cultivars in at least one experiment for each pathogen.

Mean extent of staining associated with inoculation ranged from 12-27 mm (2014-15) and 13-18 mm (2015-16) for *E. lata* and from 10-34 mm and 8-15 mm (2015-16) for *D. seriata*. Extent of staining was found to have some correlation with recovery distance for *D. seriata* (R^2 values 0.30 and 0.17 in 2014-15 and 2015-16, respectively) but no correlation for *E. lata* (Figure 10.13).

The greatest distance from the wound site that *E. lata* and *D. seriata* were recovered in single canes was 200 mm (many cultivars) and 150 mm (cv. Riesling), respectively (data not shown). The maximum distance *E. lata and D. seriata* were recovered in single canes ahead of the staining was 193 (cv. Traminer) and 94 mm (cv. Colombard), respectively.

Regression analysis between cane width (as determined in the detached cane assay) and recovery distance revealed a positive relationship with moderate coefficient of determination (R^2 values ranging from 0.24-0.42; Figure 10.14).

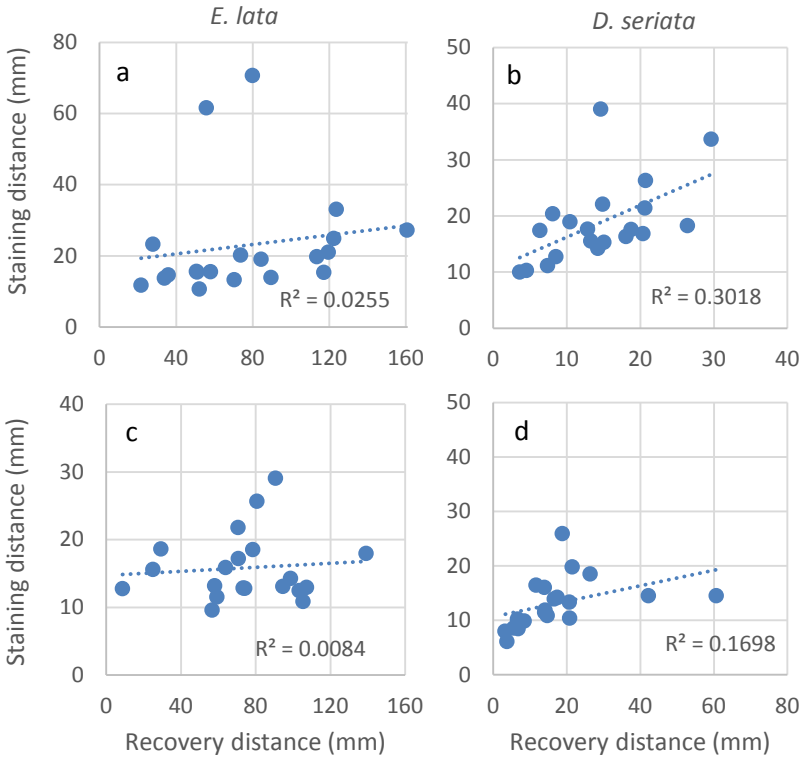


Figure 10.13 Relationship between mean distance *Eutypa lata* (a, c) and *Diplodia seriata* (b, d) were recovered from canes that were inoculated *in situ* in 2014-15 (a-b) and 2015-16 (c-d) and the distance of staining recorded on canes of cultivars in the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia.

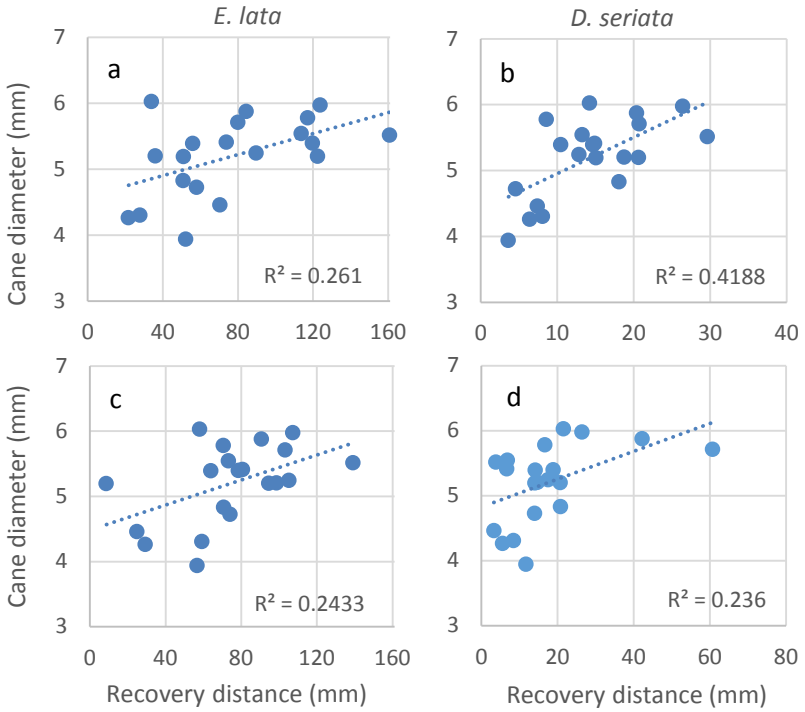


Figure 10.14 Relationship between mean distance *Eutypa lata* (a, c) and *Diplodia seriata* (b, d) were recovered from canes that were inoculated *in situ* in 2014-15 (a-b) and 2015-16 (c-d) and the diameter of detached canes of cultivars taken from the germplasm collection at the Nuriootpa Research Centre, Barossa Valley, South Australia.

10.3.2 Clonal susceptibility

The severity of trunk disease symptoms varied substantially for clones of cv. Shiraz, ranging from 0 to 73% for foliar symptoms of eutypa dieback and 28-87% for overall trunk disease symptoms of eutypa and botryosphaeria dieback (Figure 10.15). The vines ranged in age from 21 to 34 years, although the age of some clones was unknown.

The severity of trunk disease symptoms varied substantially for clones of cv. Cabernet Sauvignon, ranging from 8 to 100% for foliar symptoms of eutypa dieback and 15-100% for overall trunk disease symptoms of eutypa and botryosphaeria dieback (Figure 10.16). Age of vines ranges from 20 to 44 years, with the age of some clones unknown.

The severity of trunk disease symptoms varied substantially for clones of cv. Chardonnay, ranging from 0 to 40% for foliar symptoms of eutypa dieback and 0-63% for overall trunk disease symptoms of eutypa and botryosphaeria dieback (Figure 10.17). Age of vines ranges from 18 to 45 years, with the age of some clones unknown.

In general, for all three cultivars symptom severity also varied between vines of the same clone and younger vines tended to have fewer symptoms than the older ones in the collection.

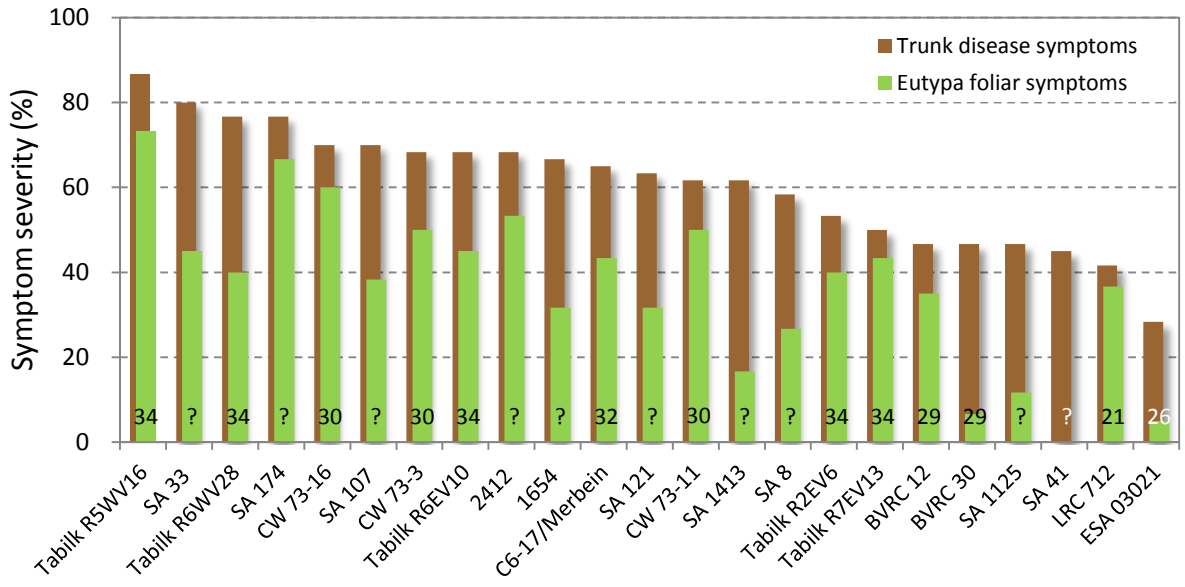


Figure 10.15 Severity of overall trunk disease symptoms of eutypa and botryosphaeria dieback (brown) and foliar symptoms of eutypa dieback (green) on clones of cv. Shiraz in the clonal collection, based on the mean assessment of three vines per clone, made in October 2014 at the Nuriootpa Research Centre, Barossa Valley, South Australia. The age of vines is indicated at the base of each column (? = unknown).

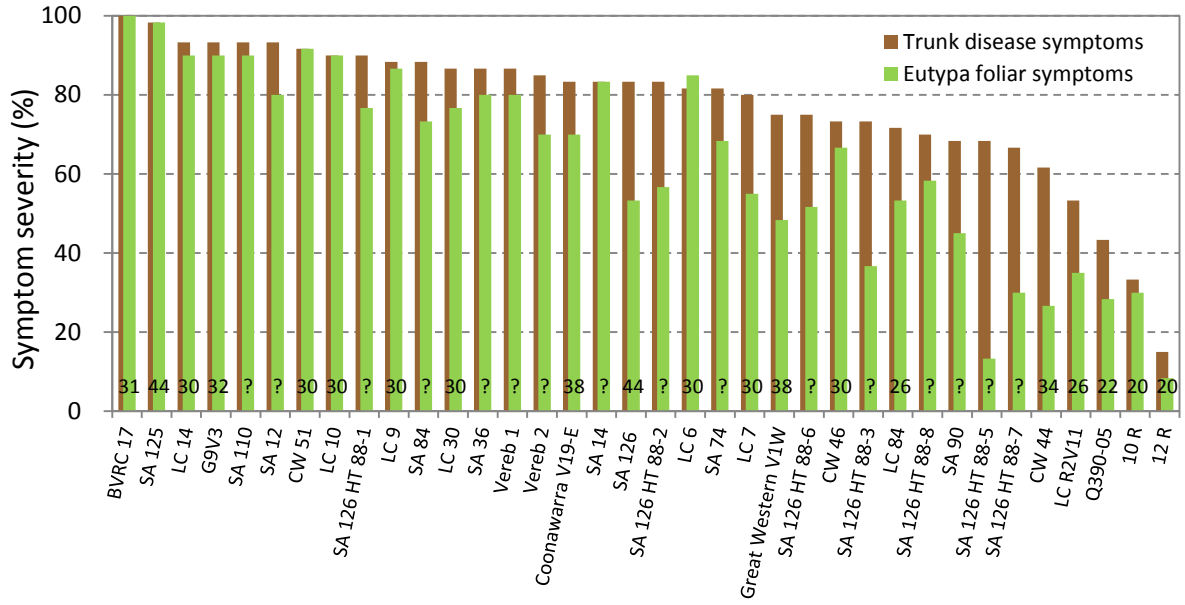


Figure 10.16 Severity of overall trunk disease symptoms of eutypa and botryosphaeria dieback (brown) and foliar symptoms of eutypa dieback (green) on clones of cv. Cabernet Sauvignon in the clonal collection, based on the mean assessment of three vines per clone, made in October 2014 at the Nuriootpa Research Centre, Barossa Valley, South Australia. The age of vines is indicated at the base of each column.

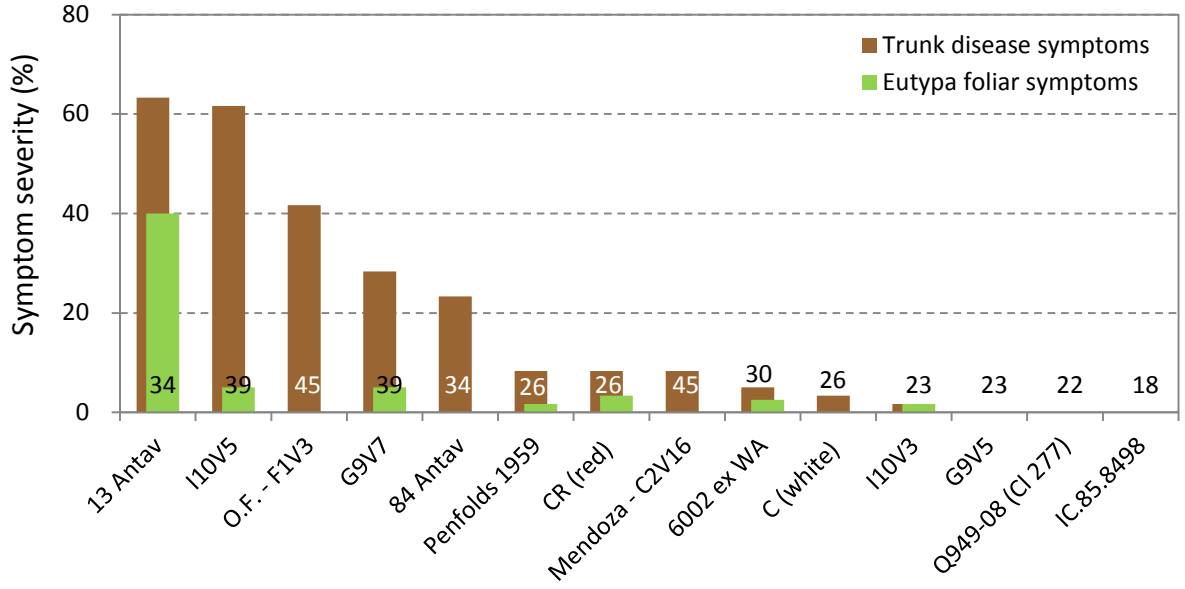


Figure 10.17 Severity of overall trunk disease symptoms of eutypa and botryosphaeria dieback (brown) and foliar symptoms of eutypa dieback (green) on clones of cv. Chardonnay in the clonal collection, based on the mean assessment of three vines per clone, made in October 2014 at the Nuriootpa Research Centre, Barossa Valley, South Australia. The age of vines is indicated at the base of each column.

10.3.3 Rootstock susceptibility

In the first set of DCA experiments, the extent of colonisation of the detached canes varied among rootstock genotypes for both pathogens (Figure 10.18a). In the susceptible cv. Shiraz, *E. lata* and *D. seriata* were recovered at 11 and 24 mm from the wound site, respectively. For the *V. vinifera* (Riesling) x *V. cinerea* genotypes, *E. lata* recovery ranged from 4-11 mm, whereas *D. seriata* recovery ranged from 12-24 mm from the wound. Recovery was significantly lower than that of cv. Shiraz for seven genotypes for *E. lata* and 12 genotypes for *D. seriata*. Six genotypes had significantly reduced recovery for both pathogens.

Cane diameter ranged from 4.1 to 7.3 mm (Figure 10.18b) and there was a weak positive correlation between *E. lata* colonisation and diameter (R^2=0.18) but no correlation for *D. seriata* (Figure 10.19).

In the second set of DCA experiments, the extent of colonisation of the detached canes varied among rootstock genotypes for both pathogens (Figure 10.20a). In the susceptible cv. Shiraz, *E. lata* and *D. seriata* were recovered at 7 and 12 mm from the wound site, respectively. For the genotypes with varying species of origin, *E. lata* recovery ranged from 7 to 20 mm, whereas *D. seriata* recovery ranged from 9 to 19 mm from the wound. Recovery was significantly more than that of cv. Shiraz for 10 genotypes for *E. lata* and one genotype for *D. seriata*, whereas no genotype exhibited significantly reduced recovery for either pathogen. There was no evidence of reduced susceptibility for any of the species evaluated.

Cane diameter ranged from 2.9 to 4.3 mm (Figure 10.20b) and there was a weak negative correlation between colonisation by both pathogens and diameter (*E. lata* R^2=0.14 and *D. seriata* R^2=0.23) (Figure 10.21).

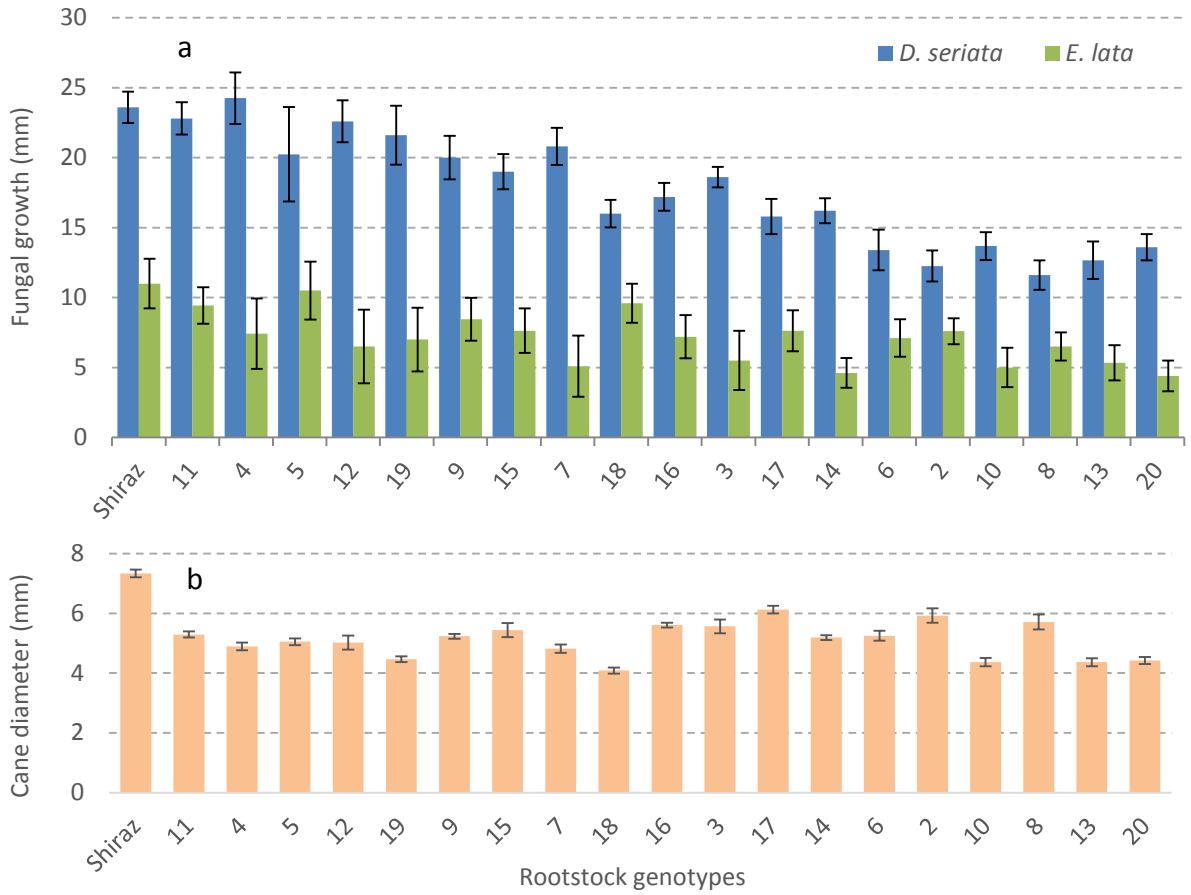


Figure 10.18 a) Mean distance of recovery from canes of 19 *Vitis vinifera* (Riesling) x *V. cinerea* rootstock genotypes (CSIRO) and cv. Shiraz by *Eutypa lata* and *Diplodia seriata* in two separate detached cane assays and b) mean width of debarked canes combined for both experiments. Bars represent standard error of the mean.

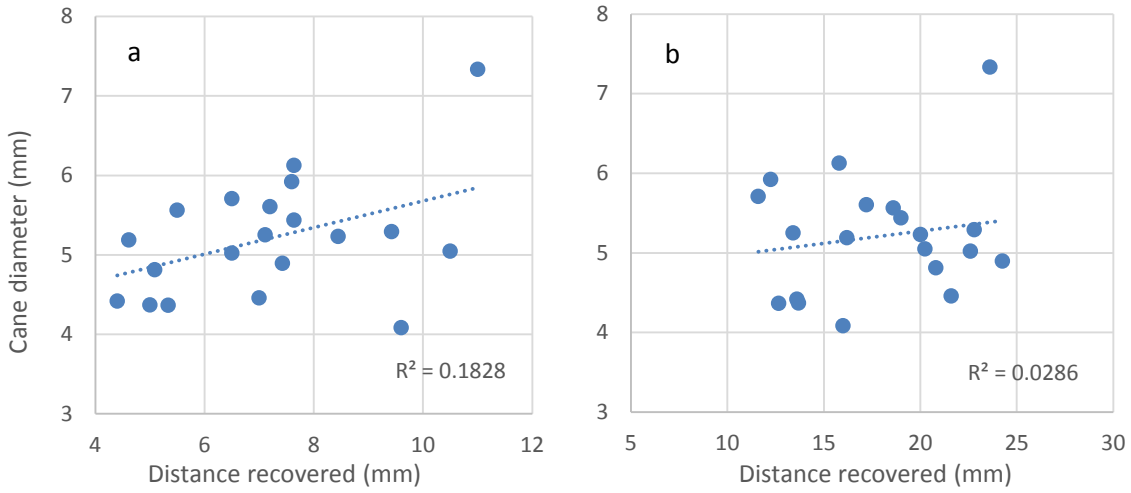


Figure 10.19 Relationship between recovery distance for *Eutypa lata* (a) and *Diplodia seriata* (b), and the width of detached canes of 19 *Vitis vinifera* (Riesling) x *V. cinerea* rootstock genotypes (CSIRO) and cv. Shiraz.

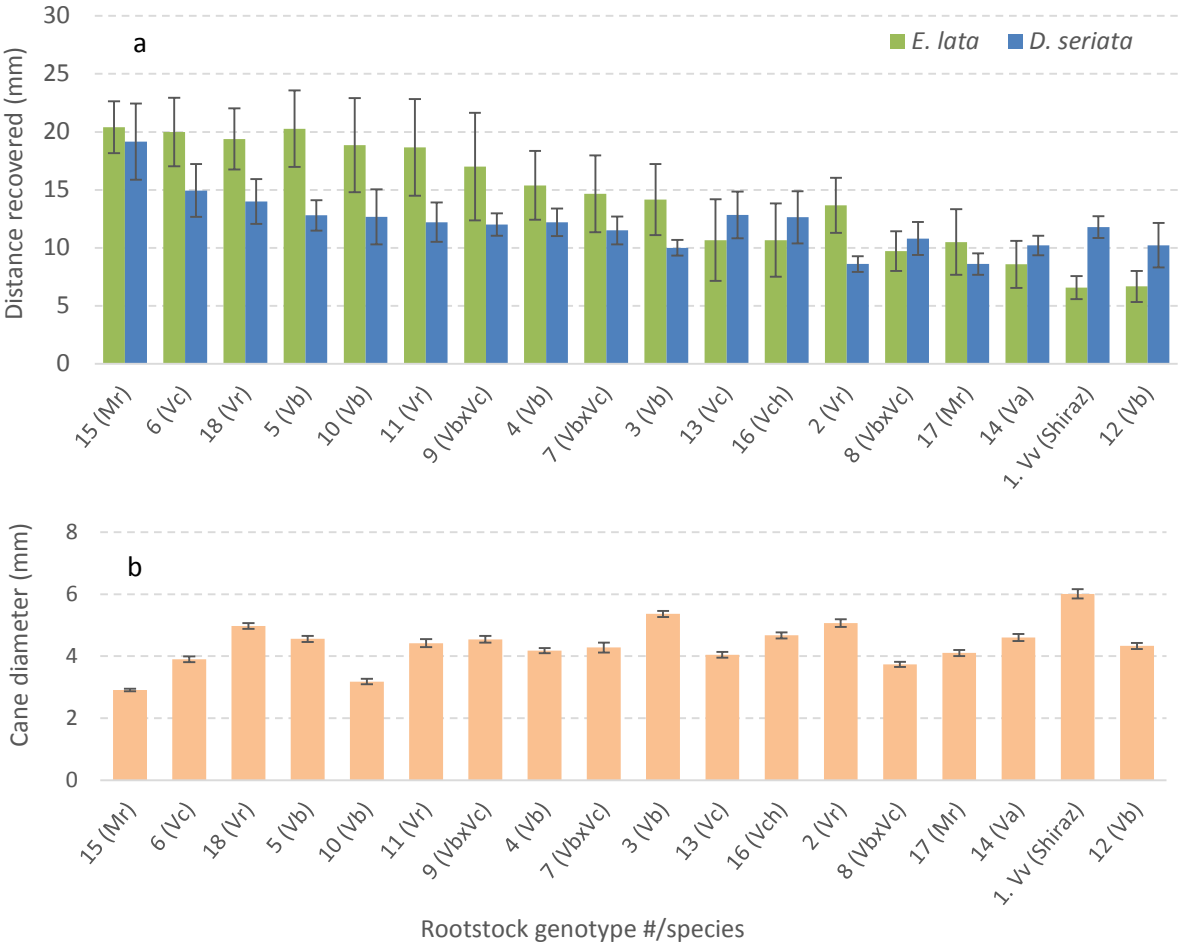


Figure 10.20 a) Mean distance of recovery from canes of 17 rootstock genotypes (CSIRO) and cv. Shiraz by *Eutypa lata* and *Diplodia seriata* in two separate detached cane assays. Bars represent standard error of the mean. Mr = *Vitis rotundifolia*, Vc = *Vitis cinerea*, Vr = *V. rupestris*, Vb = *V. berlandieri*, Vch = *V. champinii*, Va = *V. acerifolia*, Vv = *V. vinifera* and b) mean width of debarked canes combined for both experiments.

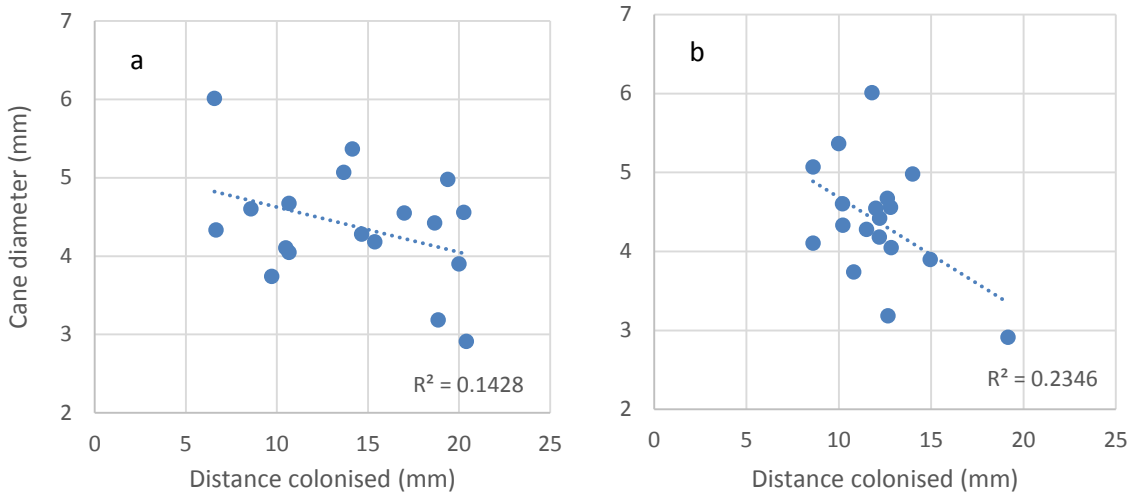


Figure 10.21 Relationship between distance recovered (a *Eutypa lata* and b *Diplodia seriata*) and the width of detached canes of 17 rootstock genotypes (CSIRO) and cv. Shiraz.

10.4 Discussion

The visual symptom assessment of the SARDI germplasm collection indicated that the severity of trunk disease varied considerably amongst the cultivars in the 31-36 year old vines. Results were generally consistent with previous reports from eutypa dieback surveys in Australia and France (Wicks 1975, Dubos 1987 cited by Carter 1991, Highet and Wicks 1998, Loschiavo *et al.* 2007). In the present study, 41 cultivars were recorded to have less than 10% severity of trunk disease, suggesting tolerance to trunk pathogens may exist. All vines were subjected to natural infection and, based on previous studies on neighbouring vines at the Nuriootpa Research Centre, up to 12% and 33% incidence of natural pruning wound infection has been reported for eutypa and botryosphaeria dieback pathogens, respectively (Sosnowski *et al.* 2008, Pitt *et al*. 2012, Sosnowski *et al.* 2013). However, fungal isolations from vines in the germplasm collection will be required to confirm the cause of symptoms, and whether species responsible for botryosphaeria dieback are also involved.

Following the assessment of symptoms in the field, 18 of the least symptomatic cultivars were selected for further evaluation using detached canes, potted vines and by inoculation of pruning wounds on field vines, and compared with the susceptible cultivars, Shiraz and Sauvignon Blanc to seek cultivars with potential tolerance to GTD pathogens.

Results from the DCA experiments indicated that susceptibility to the pathogens *E. lata* and *D. seriata* differs amongst cultivars. Seven cultivars were consistently less susceptible to *E. lata* than Shiraz and Sauvignon Blanc, with Petit Meslier and Auxerrois showing the most potential for tolerance to eutypa dieback. Two cultivars, Green Veltliner and Muscadelle, were consistently less susceptible to *D. seriata* than Shiraz and Sauvignon Blanc, thereby showing the most potential for tolerance to botryosphaeria dieback. However, for some cultivars, the ranking of cultivars tended to vary considerably between repeated experiments. For example, Limberger and Traminer ranked differently for *E. lata* and Muscat a Petit Grains Rouge, Pinot Noir and Sangiovese changed in ranking for *D. seriata*. Therefore it is difficult to draw conclusions on their potential for tolerance to GTD pathogens. For the potted vine experiments, in order to reduce the variability experienced when using spores liberated from fruiting bodies on dead wood, a novel method of wound inoculation (Travadon *et al.* 2013) using a slurry of mycelial fragments was adopted to allow the use of a single isolate of *E. lata*. However, *E. lata* was recovered infrequently, leading to insufficient data for analysis. For the *D. seriata* experiment, in which the mycelial slurry inoculation method was adopted also, results differed greatly to those obtained from the DCA experiments, and the susceptible Sauvignon Blanc was ranked amongst the cultivars with the smallest pathogen colonisation distance in the stems of the potted vines. The experiment may have been compromised by the use of mycelial inoculation method, which does not reflect natural infection arising from spores of the pathogens.

Inoculation of pruning wounds on canes *in situ* in the SARDI germplasm collection confirmed the variation in susceptibility of cultivars to infection by *E. lata* and *D. seriata*. Petit Verdot and Muscadelle cultivars were consistently the least colonised by both pathogens, indicating that, of all

cultivars evaluated, they are likely to have the greatest tolerance to infection. In at least one of the field experiments, the Muscat a Petit Grains Rouge, Ughetta, Pinot Noir and Riesling were colonised by each pathogen significantly less than the susceptible cultivars, suggesting potential tolerance to eutypa and botryosphaeria dieback. Furthermore, Petit Meslier and Melon were colonised significantly less by *D. seriata* than the susceptible cultivars, suggesting potential tolerance to botryosphaeria dieback only.

Results from inoculations in the vineyard were not always consistent with those in the detached cane assay. This may be due to physiological or biochemical changes in the tissues of canes once detached from the vine, potentially altering their natural defence mechanisms. Also, the short incubation period of four weeks used for detached canes compared with 12 months in the vineyard may have limited the ability of the mycelial growth to progress as it would over the longer period. Although the incidence of infection by *E. lata* has been shown to be consistent in attached and detached canes when evaluating wound treatments (Ayres *et al*. 2017), similar comparisons for rate of colonisation in canes have not been reported previously. The results obtained here suggest that the detached cane assay may not be a reliable method for evaluating the growth rate of trunk pathogens in living vines, but this requires further investigation.

The visual assessment of symptoms in the SARDI clonal collection indicated that the severity of trunk disease varied considerably amongst the clones of Shiraz, Cabernet Sauvignon and Chardonnay. Caution should be taken when interpreting these results as they are based on a single visual assessment and may be influenced by the effect of vine age, which differed amongst the clones in the collection. However, it provides enough evidence to warrant further investigation to determine whether certain clones might be recommended for future plantings.

The rootstock genotypes examined in the DCA varied in their susceptibility to colonisation by *E. lata* and *D. seriata*. Several genotypes in the CSIRO collection of *V. vinifera* (Riesling) x *V. cinerea* genotypes were colonised to a lesser extent than cv. Shiraz in detached cane assays. Further evaluation of these and other genotypes with similar parentage is warranted and may lead to the eventual production of rootstocks with reduced susceptibility to trunk diseases. Based on results for the cultivars in the germplasm collection, it may be necessary to evaluate rootstocks further by inoculating canes on vines in the field or providing a longer incubation period in the detached canes.

There was some correlation between cane width and colonisation by *E. lata* and *D. seriata*, particularly for the *V. vinifera* cultivars. In a recent Honours study, Hamblin (2015) further evaluated some cultivars with low susceptibility to *E. lata* based on the current research and found that some had smaller cane and xylem vessel diameter and greater lignin content than susceptible cultivars, which was consistent with findings by Rolshausen *et al.* (2008) and Pouzelet *et al.* (2014). These traits may be useful markers when selecting for tolerant cultivars or new genotypes.

In the field inoculations, *E. lata* colonised canes to a significantly greater extent than *D. seriata*, although differences were smaller and results more variable in the detached cane assays. This contradicts previous understanding that *D. seriata* was similar in pathogenicity to *E. lata* (Pitt *et al*. 2013). However, the previous study was based on necrotic staining in the wood and, as was observed for both pathogens in the experiments here with both pathogens and previously reported for *E. lata* (Sosnowski *et al.* 2007b), the fungus can grow ahead of the stained wood in apparently healthy wood tissue, with no correlation between staining and pathogen recovery. This casts doubt over the usefulness of extent of staining as an indicator of disease severity.

In the present study, the pathogens were recovered up to 193 mm (*E. lata*) and 94 mm (*D. seriata*) ahead of staining in individual instances, compared with 80 mm in the previous study involving *E. lata* in several different cultivars (Sosnowski *et al.* 2007b), which suggests that the current recommendation of cutting at least 10 cm beyond any stained wood during remedial surgery may be insufficient to ensure that the fungus is removed. Also shown in the present work, *E. lata* and *D. seriata* colonised canes to a distance of more than 200 and 150 mm, respectively, below the wound surface over 12 months, which is further than the 115 mm previously reported for *E. lata* (Sosnowski *et al.* 2007b). Therefore, further research is required to assess a greater distance along canes and determine the maximum extent of colonisation of canes in a range of cultivars and under a variety of environmental conditions over 12 months. This would provide greater confidence in pruning management strategies that remove infected tissues from vines.

11 WATER STRESS

11.1 Introduction

Environmental conditions are thought to be an important factor in the expression of foliar symptoms of eutypa dieback, and variation in severity of symptoms has been recorded from year to year in Australia (Creaser and Wicks 2001, Sosnowski *et al*., 2007a), France (Dumot *et al*., 2004) and the USA (Butterworth *et al*., 2005). Sosnowski *et al*. (2007a) reported an increase in symptom expression to be associated with higher winter rainfall and suggested that increased water availability may facilitate transport of toxins to the foliage in spring. Furthermore, they also reported that decreased disease incidence was associated with increased temperature in spring. As vines grow more vigorously in warmer conditions, it was proposed that the ability of fungal toxins to reach the foliage might be reduced and the increased amount of foliage may dilute the toxins. In a subsequent controlled environment experiment, potted vines subjected to a combination of extreme heat or cold plus low or high soil moisture displayed more severe foliar symptoms than those in moderate conditions (Sosnowski *et al.* 2011a).

Research has also demonstrated that low soil water content (Levitt, 1980, Smart and Coombe, 1983), and high temperature (Kriedemann and Smart, 1971) can compromise grapevine health by influencing the severity of other diseases. Environment and plant health, therefore, may influence the expression of foliar symptoms of eutypa dieback and the growth rate of the pathogen.

Regulated deficit irrigation (RDI) involves the control and management of water stress by irrigating at less than the full requirement of the vines and maintaining soil moisture at a relatively low level, to control vigour and increase the fruit quality while conserving water (McCarthy *et al*., 2002). Furthermore, climate change may lead to more extreme environmental conditions for grape growing in the future, especially via drought, by increasing water stress in the summer months (Schultz, 2000; Jones, 2005). Spores of *E. lata* infect vines through pruning wounds, and the natural wound protection response of the vine plays a role in reducing the ability of the fungus to colonise the tissue. Sosnowski *et al.* (2011) reported that water-stressed vines in a warm, dry environment were more susceptible to infection of pruning wounds by *E. lata* than vines receiving standard watering.

The aim of this study was to further evaluate the ability of *Eutypa lata* (eutypa dieback) and *Diplodia seriata* (botryosphaeria dieback) to infect wounds and colonise canes on vines subjected to different levels of irrigation and water stress.

11.2 Methods

Two trials were established in South Australia, at the Loxton Research Centre in the Riverland and Nuriootpa Research Centre in the Barossa Valley, to investigate the effect of water deficit irrigation on the response of grapevines to inoculation with trunk disease pathogens.

In the Riverland, Shiraz vines grafted on Ramsey rootstock (planted in 1992) and top-worked to Cabernet Sauvignon in 2010 were subjected to four irrigation levels; 100, 50, 25 and 12.5% of the standard irrigation program using under-vine Rotor Spray™ sprinklers of different size (Antelco, Murray Bridge, SA; Figure 11.1a), beginning in December 2011. The trial was established as a randomised block design with four replications, three panels (two vines per panel) x three rows for each irrigation treatment, and the four vines in the centre used for inoculations, leaving a buffer zone between treatments. Vines were spur-pruned with four cordons (Figure 11.1b).

In the Barossa Valley, Shiraz (clone BVRC 30) vines planted in 2004 either on own roots, or grafted onto rootstocks; 1103 Paulsen, 99 Richter, 110 Richter and 140 Ruggeri (*Vitis berlandieri* x *V. rupestris*), Schwarzmann (*V. riparia* x *V. rupestris*) or Ramsey (*V. champinii*), were either drip-irrigated fully (100% of the allocation in each year) or not irrigated at all (0%), beginning in 2008. The trial was designed as a split-plot, with main plots (rootstocks) and sub-plots (irrigation treatment) with three replications. Irrigation treatments were applied to four panels (three vines per panel) x three rows with the three vines in the centre used for inoculations, leaving a buffer zone between irrigation treatments. Vines were spur-pruned with two cordons.

Daily and long term average weather data for the two locations were provided by the Bureau of Meteorology. Mean annual rainfall in the Riverland and Barossa Valley are 261 and 472 mm, and mean annual maximum temperature 23.8 and 21.5ºC, respectively.

One-year-old shoots were pruned to approx. 30 cm spurs (3-5 buds) on 9 and 10 July 2013 in the Riverland and Barossa Valley, respectively. Within an hour of pruning, wounds were moistened by spraying with sterile distilled water (SDW) immediately prior to inoculation with approximately 500 spores of *E. lata* or 1000 spores of *D. seriata* suspended in 20 µL SDW and applied using a pipette. Spore suspensions were produced using methods described in Section 7.2.1. In the Riverland, 20 canes per four vine plot vine were inoculated with each pathogen and a further 20 canes were left as non-inoculated controls. In the Barossa Valley, 10 canes per vine (one vine per pathogen) were inoculated and a vine with 10 canes was left as a non-inoculated control.

The trials were repeated in 2014 with pruning occurring on 8 and 9 July in the Riverland and Barossa Valley, respectively.

To determine the extent to which *E. lata* and *D. seriata* colonised the canes, the entire canes were removed from vines on 8 and 9 July 2014 and 10 July and 18 June 2015 for the Riverland and Barossa Valley trials, respectively, and placed in cool storage. For assessment, bark was removed from canes using a sharp knife and the canes were surface sterilised in 2.5% sodium hypochlorite for 10 minutes followed by rinsing in SDW. Wood pieces (~2 mm discs) were cut with sterile secateurs at 10 mm intervals up to 200 mm below the inoculation point and transferred to agar plates. Following 7-10 days of incubation at 23ºC under fluorescent light, cultures were identified using colony morphology, and the distances at which fungi were reisolated below the wound surface recorded. 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.



Figure 11.1 Deficit irrigation trial at Loxton in the Riverland SA, (a) Rotor Spray™ under-vine sprinkler and (b) four-cordon spur-pruned vines with treated canes tagged.

Leaf water potential

Grapevine leaves were analysed using a pressure chamber on 23 and 29 January, 2014 and 29 January and 5 February 2015, in the Barossa Valley and Riverland, respectively, to give an estimation of soil water tension, based on the method described by Canny and Roderick (2005). The youngest fully formed leaf, including petiole, was picked from a shoot from one vine per treatment for each replication. The leaf was placed in a snap-lock bag with the petiole protruding, which was then cut at a 45° angle with a razor blade. The petiole was then placed through a grommet in the pressure chamber cap which was tightened to prevent the escape of gas. The cap was secured on the pressure chamber and the nitrogen gas cylinder valve was opened, allowing pressurisation of the chamber. The pressure in the chamber was slowly increased, whilst watching the protruding end of

the petiole. When the first bubbles were observed from the petiole, the pressure reading was recorded. This was repeated two to three times per treatment over a 4 to 6 hour period between midnight and dawn. The means of the leaf water potential (LWP) readings were recorded.

11.3 Results

11.3.1 Riverland trial

In the Riverland trial, the incidence of *E. lata* recovery from wounds tended to decrease with increasing LWP, but the difference was statistically significant only for 100 and 12.5% irrigation in 2013-14. Recovery of *E. lata* ranged from 74 to 92% in 2013-14 and 68 to 82% in 2014-15 (Figure 11.2). Incidence of *D. seriata* recovery did not differ between irrigation treatments in either year, ranging from 86-93% recovery in 2013-14 and 68-71% recovery 2014-15. Non-inoculated control wounds were recorded with 2% (2013-14) and 8% (2014-15) recovery of either pathogen (Table 11.1).

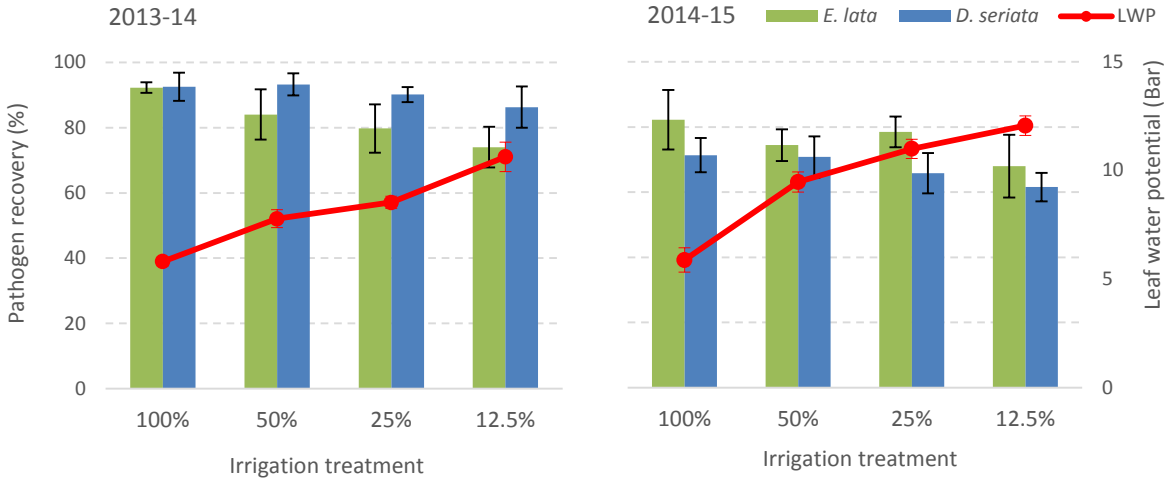


Figure 11.2. Effect of irrigation treatment on recovery incidence of *Eutypa lata* (green columns) and *Diplodia seriata* (blue columns) from pruning wounds following inoculation of canes in a trial repeated over two years on cv. Cabernet Sauvignon at the Loxton Research Centre in the Riverland, South Australia. Red line represents the mean leaf water potential (LWP). Bars represent standard error of the mean.

Mean distance of recovery, for all treatments combined, differed between trials over the two years for *E. lata* (88 and 71 mm in 2013-14 and 2014-15, respectively) and *D. seriata* (44 and 27 mm, respectively; Table 11.1). Therefore, trials were statistically analysed and are presented separately below. For some individual canes, the distance at which both pathogens were recovered from the wound site exceeded 200 mm, which was the furthest distance at which the tissue was assessed.

In 2013-14, LWP was 5.8 Bar for the full irrigation treatment and increased significantly to 10.7 Bar for the lowest irrigation treatment (12.5%; Figure 11.3). The overall mean distance of recovery was significantly greater for *E. lata* (88 mm) than for *D. seriata* (44 mm). The greatest colonisation of canes by *E. lata* occurred in vines with full irrigation, with mean recovery distance of 106 mm from the wound site (Figure 11.3). The mean recovery distance decreased significantly to 89 mm for the 25% irrigation treatment and 60 mm for 12.5% irrigation treatment. There was no difference in recovery distance of *N. luteum* between the irrigation treatments (Figure 11.3).

In 2014-15, LWP was 5.9 Bar for the full irrigation treatment and increased significantly to 12.1 Bar for the lowest irrigation treatment (12.5%; Figure 11.3). The overall mean distance of recovery was significantly greater for *E. lata* (71 mm) than for *D. seriata* (27 mm). The greatest colonisation of canes by *E. lata* occurred in vines with full irrigation, with mean recovery distance of 80 mm from the wound site (Figure 11.3), significantly further than that recorded from canes on vines with 12.5% irrigation (61 mm). There was no difference in recovery of *N. luteum* between the irrigation treatments.

Regression analysis of data from both years revealed a negative correlation (R^2=0.61) between LWP and recovery distance of *E. lata* (Figure 11.4a), but no correlation (R^2=0.02) between LWP and recovery distance of *D. seriata* (Figure 11.4b).

In non-inoculated controls, colonisation of canes by either pathogen did not exceed a mean of 3.7 mm (2013-14) or 4.5 mm (2014-15) for any of the treatments (Figure 11.3).

In both years, the extent of staining of canes did not differ between pathogens or irrigation treatments, with mean distances ranging from 15-25 mm (2013-14) and 13-20 mm (2014-15; Figure 11.3). There was little correlation between recovery of *E. lata* (R^2=0.26) and *D. seriata* (R^2=0.35) and the staining (Figure 11.4c-d). Non-inoculated controls had similar extent of staining, ranging from 14-19 mm (2013-14) and 18-22 mm (2014-15). The mean distance at which pathogens were recovered from canes ahead of the staining ranged from 39-86 mm (*E. lata*) and 5-26 mm (*D. seriata*). The maximum distance at which the pathogen was recovered ahead of the staining for an individual cane was 196 mm (*E. lata*) and 192 mm (*D. seriata*).

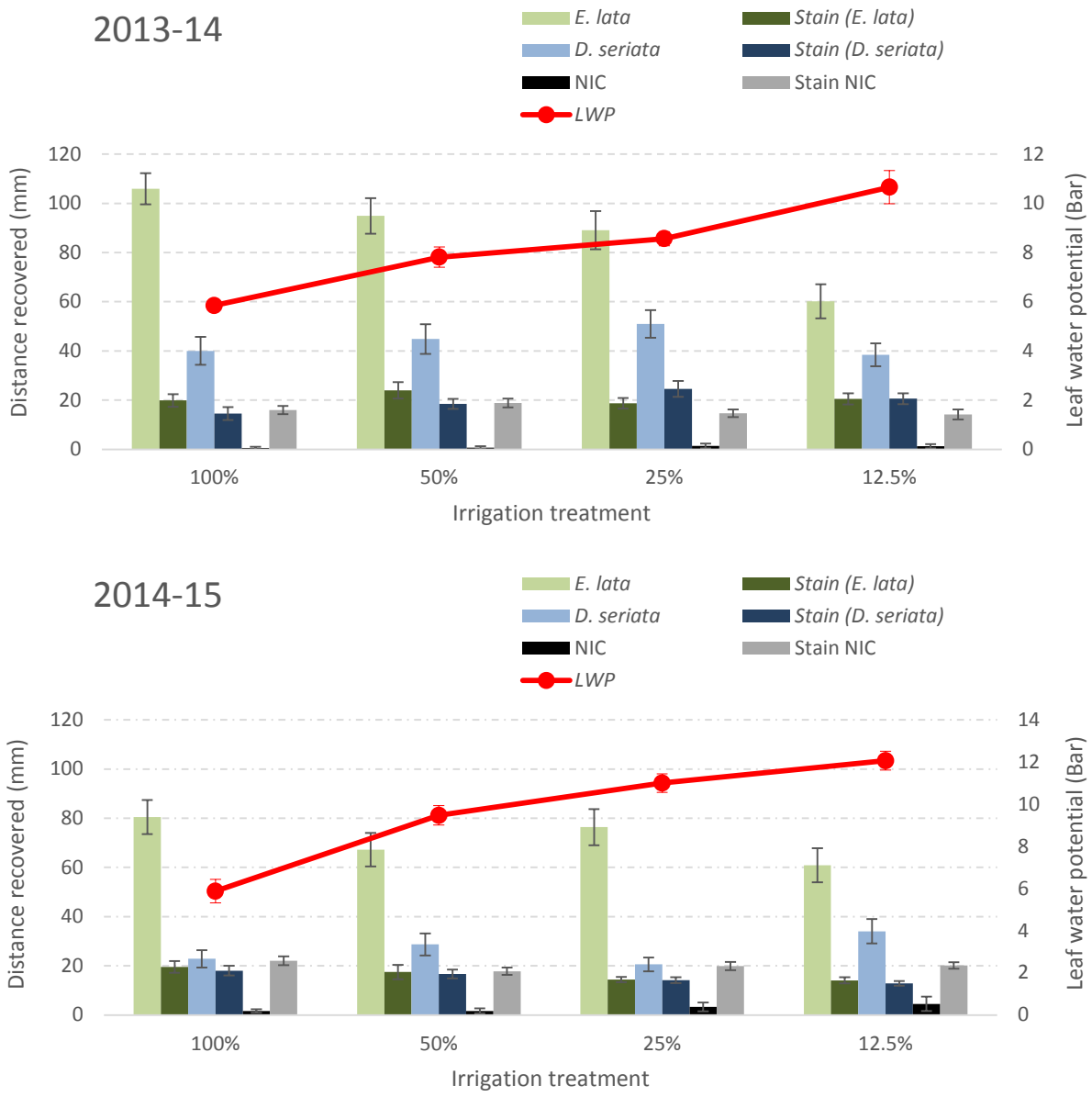


Figure 11.3. Effect of irrigation treatment on recovery distance of *Eutypa lata* (light green columns) and *Diplodia seriata* (light blue columns) from pruning wounds following inoculation of canes, and staining (dark green and blue columns, respectively) in trial repeated over two years on cv. Cabernet Sauvignon at the Loxton Research Centre in the Riverland, South Australia. Recovery distance of either pathogen from non-inoculated controls (NIC) is indicated by black columns, and staining in grey columns. Red line represents the mean leaf water potential (LWP). Bars represent standard error of the mean.

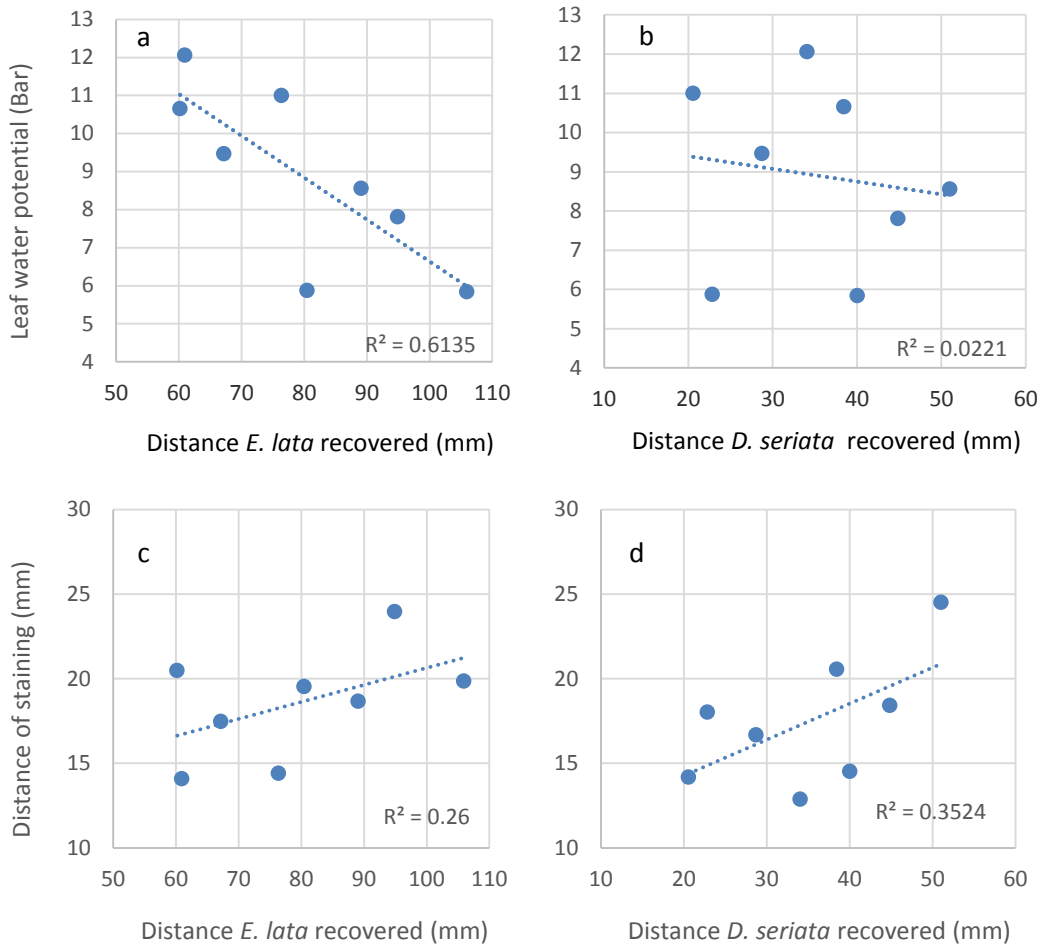


Figure 11.4 Relationship between distance at which pathogen recovered (*Eutypa lata* and *Diplodia seriata*) from inoculated canes and the leaf water potential (a-b) or distance of staining (c-d) on cv. Cabernet Sauvignon at the Loxton Research Centre in the Riverland, South Australia.

Climatic conditions around the time of pruning and inoculation varied between the years (Table 11.1). During the week following pruning, temperature, relative humidity and solar radiation were greater in 2013-14 compared with 2014-15. There was no rainfall recorded in the two days before and after pruning in 2013-14, whereas 1.6 mm fell during the same period in 2014-15. However, more rain fell seven days prior to and 7-14 days post pruning in 2013-14, compared with the same periods in 2014-15.

Table 11.1. Recovery incidence and distance of *Eutypa lata* (El) and *Diplodia seriata* (Ds) from cv. Cabernet Sauvignon canes for all treatments combined of inoculated (IC) and non-inoculated (NIC) controls in 2013-14 and 2014-15, along with climate variables of mean maximum temperature, mean relative humidity (RH), solar radiation and cumulative rainfall, for days before (-) and after pruning at the Loxton Research Centre in the Riverland, South Australia.

	IC - El		IC - Ds		NIC		Max Temp (°C)			RH (%)			Solar (W/m^2) (x1000)			Rainfall (mm)					
	%	mm	%	mm	%	mm	1 d	2 d	7 d	1 d	2 d	7 d	1 d	2 d	7 d	-7 d	-2 d	1 d	2 d	7 d	14 d
2013-14	83	88	91	43	2	1	14	16	17	83	82	86	13.7	12.7	8.7	2.2	0	0	0	6.2	22
2014-15	76	71	67	27	8	3	11	13	13	80	80	81	7.9	8.9	9.6	0.8	0.2	1.2	1.4	2.4	2.8

11.3.2 Barossa Valley trial

Over the two years of the Barossa Valley trial, the overall incidence of recovery from wounds was generally greater in 2013-14 than in 2014-15 and greater for *D. seriata* than for *E. lata* (Figure 11.5). However, over the two years, incidence of recovery did not differ significantly between rootstock or irrigation treatments, and ranged from 60-97% (*E. lata*) and 86-100% (*D. seriata*) in 2013-14 and from 48-87% (*E. lata*) and 61-93% (*D. seriata*) in 2014-15. Non-inoculated control wounds were recorded with 10% (2013-14) and 15% (2014-15) recovery of either pathogen (Table 11.2).

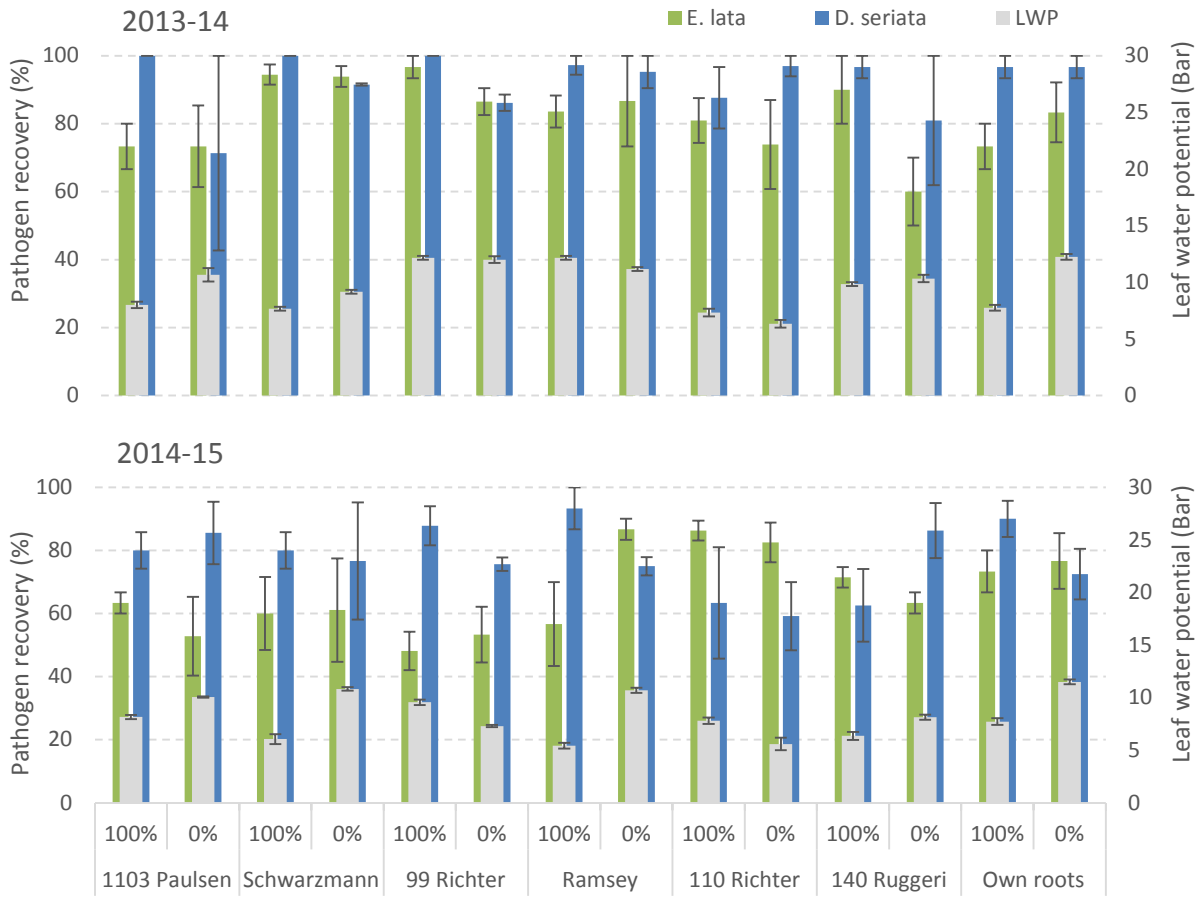


Figure 11.5. Effect of rootstock and irrigation treatment on recovery incidence of *Eutypa lata* (green columns) and *D. seriata* (blue columns) from pruning wounds of cv. Shiraz (clone BVRC 30) at the Nuriootpa Research Centre in the Barossa Valley, South Australia. Grey columns represent the mean leaf water potential (LWP) and bars represent standard error of the mean.

Mean distance of recovery differed between pathogens and trials over the two years, with mean recovery of *E. lata* (89 and 40 mm) and *D. seriata* (33 and 23 mm) in 2013-14 and 2014-15, respectively (Table 11.2). Non-inoculated control wounds were recorded with 2% (2013-14) and 16% (2014-15) recovery of either pathogen. Therefore, trials and pathogens were statistically analysed and presented separately. For some individual canes, the distance *E. lata* was recovered from the wound site exceeded 200 mm, which was the furthest distance that the tissue was assessed.

Overall, *E. lata* colonised canes further in vines with full irrigation (96 and 45 mm in 2013-14 and 2014-15, respectively) than in non-irrigated vines (78 and 35 mm). There was a significant interaction between rootstocks and irrigation treatment, with recovery of *E. lata* ranging from 24-140 and 17-59 mm from the wound site in 2013-14 and 2014-15, respectively (Figure 11.6). For the rootstocks; 1103 Paulsen, Schwarzmann and 99 Richter, as well as own-rooted vines, there was no effect of irrigation treatment on colonisation of the scion canes over both years of the trial. For the rootstocks 110 Richter and 140 Ruggeri, *E. lata* was recovered significantly further from the wound site in scion

canes on the fully irrigated than non-irrigated vines. For the rootstock Ramsey, *E. lata* was recovered significantly further from the wound site on scion canes in non-irrigated than in irrigated vines.

Mean distance of staining on canes inoculated with *E. lata* ranged from 13-36 and 13-30 mm in 2013-14 and 2014-15, respectively (Figure 11.6). Non-inoculated canes were recorded to have stained wood at mean distances of 11-92 and 10-59 mm in 2013-14 and 2014-15 below the wound surface, respectively (data not shown). *Eutypa lata* was recovered, on average, ahead of the staining for all rootstock and irrigation treatments, ranging from 9-126 and 0.2-35 mm in 2013-14 and 2014-15, respectively. The maximum distance at which *E. lata* was recovered ahead of the staining for an individual cane was 198 mm. There was no correlation between distance at which *E. lata* was recovered and extent of staining (R^2=0.04; Figure 11.7a).

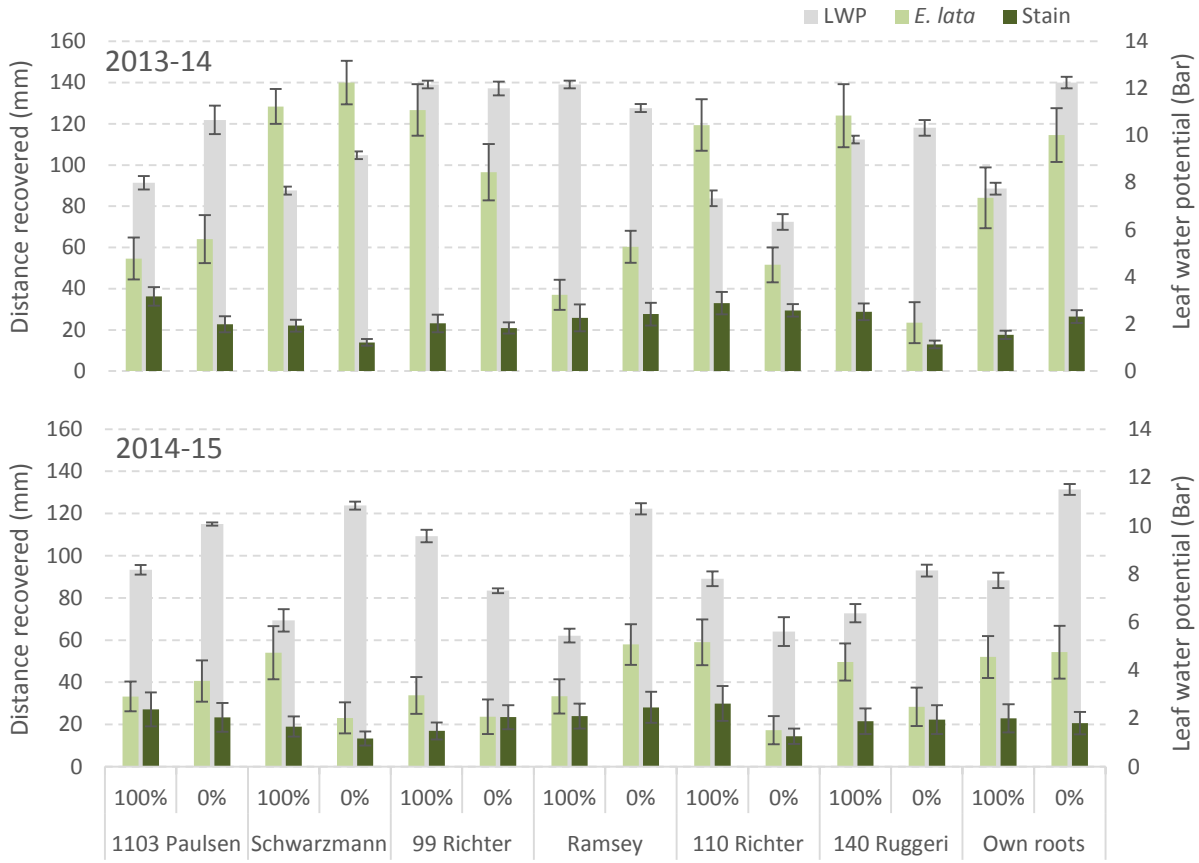


Figure 11.6. Effect of rootstock and irrigation treatment on susceptibility of cv. Shiraz (clone BVRC 30) pruning wounds to colonisation by *Eutypa lata* (light green columns) and associated staining (dark green columns) at the Nuriootpa Research Centre in the Barossa Valley, South Australia. Grey columns represent the mean leaf water potential (LWP) and bars represent standard error of the mean.

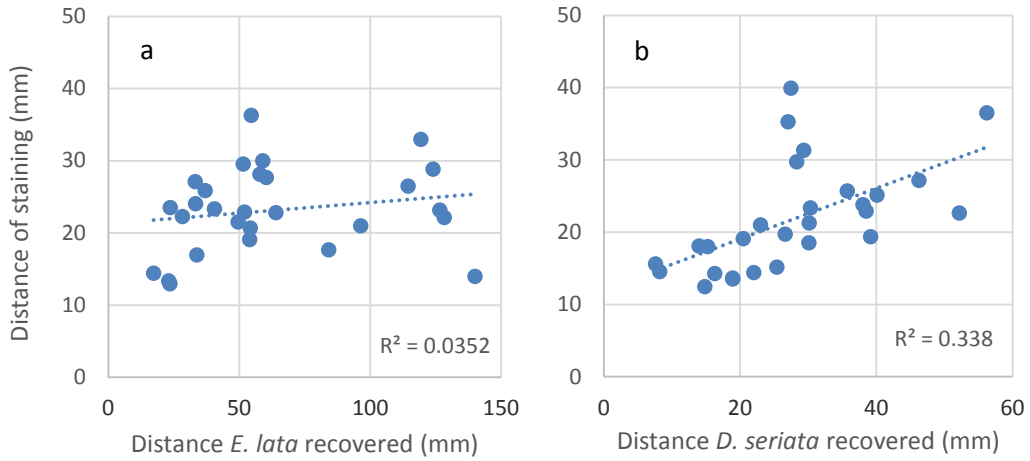
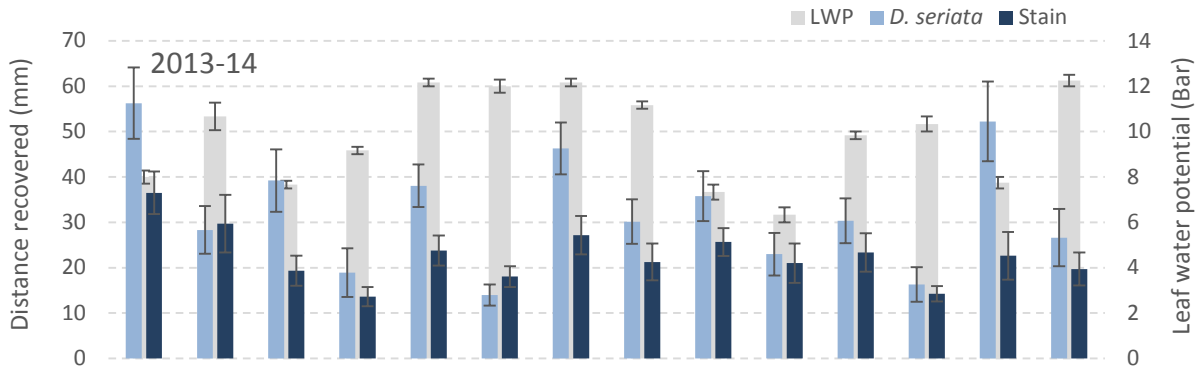


Figure 11.7 Relationship between distance at which pathogen recovered (a *Eutypa lata* and b *Diplodia seriata*) from inoculated canes and the distance of staining across all rootstocks and irrigation treatments for both trials on cv. Shiraz (clone BVRC 30) at the Nuriootpa Research Centre in the Barossa Valley, South Australia.

Overall, *D. seriata* colonised canes further in vines with full irrigation (43 and 30 mm in 2013-14 and 2014-15, respectively) than in non-irrigated vines (22 and 17 mm). For individual canes, the maximum distance *D. seriata* was recovered from the wound site exceeded 200 mm, which was the furthest distance that the tissue was assessed. There was no interaction between rootstocks and irrigation treatment, with recovery of *D. seriata* ranging from 14-56 and 8-40 mm from the wound site in 2013-14 and 2014-15, respectively (Figure 11.8). For the rootstocks; Schwarzmann, 99 Richter, Ramsey and 110 Richter, as well as own rooted vines, *D. seriata* was recovered significantly further from the wound site in canes on the fully irrigated than non-irrigated vines. For the rootstocks 1103 Paulsen and 140 Ruggeri, *D. seriata* was recovered further from the wound site of canes on the scion in fully irrigated than in non-irrigated vines in 2013-14, but not in 2014-15.

Mean extent of staining on canes inoculated with *D. seriata* ranged from 14-37 and 13-40 mm in 2013-14 and 2014-15, respectively (Figure 11.8). *Diplodia seriata* was recovered ahead of the staining for most rootstock and irrigation treatments, ranging from 2-30 and 1-15 mm in 2013-14 and 2014-15, respectively. There were a number of treatments where, on average, staining extended further than the pathogen by 1-4 and 2-12 mm in 2013-14 and 2014-15, respectively. Individual canes were recorded to yield *D. seriata* at between 0 and 177 mm ahead of staining. There was a weak correlation between distance at which *D. seriata* was recovered and staining distance (R^2=0.34; Figure 11.7b).



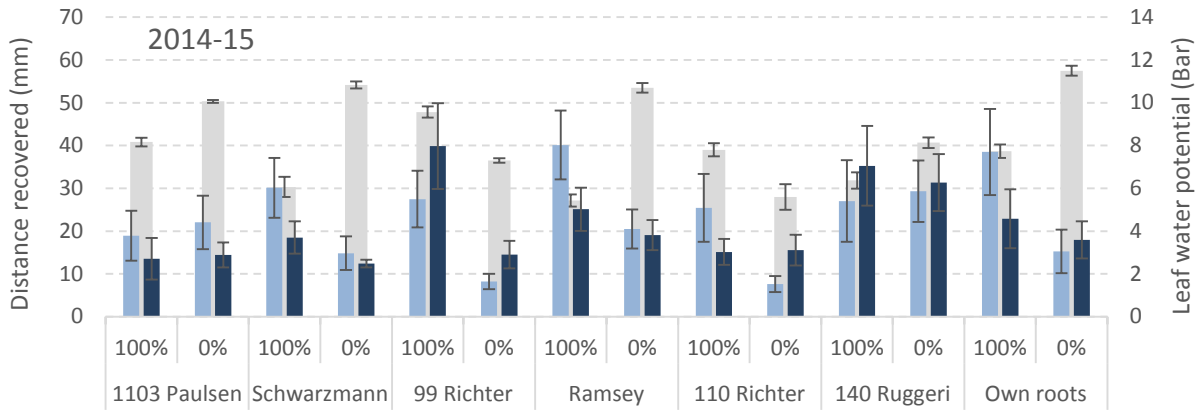


Figure 11.8. Effect of rootstock and irrigation treatment on susceptibility of cv. Shiraz (clone BVRC 30) pruning wounds to colonisation by *Diplodia seriata* (light blue columns) and associated staining (dark blue columns) at the Nuriootpa Research Centre in the Barossa Valley, South Australia. Grey columns represent the mean leaf water potential (LWP) and bars represent standard error of the mean.

There was a significant interaction effect between rootstock and irrigation treatment for LWP in both years. LWP ranged from 6.3-12.3 Bar (2013-14) and 5.6-11.5 Bar (2014-15; Figure 11.6) For the rootstocks; 1103 Paulsen, Schwarzmann and 140 Ruggeri, as well as own-rooted vines, leaves on the cv. Shiraz vines had higher LWP when not irrigated than when irrigated. Conversely, for the rootstock 110 Richter, LWP was higher in irrigated than non-irrigated vines. The rootstocks 99 Richter and Ramsey varied in LWP for the two irrigation treatments over the two years. There was no correlation between LWP and recovery distance for either pathogen (data not shown).

Climatic conditions around the time of pruning and inoculation varied between the years (Table 11.2). During the week following pruning, temperature was greater in 2013-14 compared with 2014-15, whereas relative humidity was greater in 2014-15, particularly during the first two days following pruning. There was no rainfall recorded in the two days after pruning in 2013-14, whereas 21 mm fell during the same period in 2014-15. Similar rainfall was recorded seven days prior to pruning in both years, but more rain fell 14 days post pruning in 2013-14 than during the same period in 2014-15.

Table 11.2. Recovery incidence and distance of *Eutypa lata* (EI) and *Diplodia seriata* (Ds) from cv. Shiraz canes for all treatments combined of inoculated (IC) and non-inoculated (NIC) controls in 2013-14 and 2014-15, along with climate variables of mean maximum temperature, mean relative humidity (RH) and cumulative rainfall, for days before (-) and after pruning at the Nuriootpa Research Centre in the Barossa Valley, South Australia.

	IC - EI		IC - Ds		NIC		Max Temp (°C)			RH (%)			Rainfall (mm)					
	%	mm	%	mm	%	mm	1 d	2 d	7 d	1 d	2 d	7 d	-7 d	-2 d	1 d	2 d	7 d	14 d
2013-14	83	89	93	33	10	2	13	15	15	71	70	83	13	0.2	0	0	20	57
2014-15	67	40	78	23	15	16	9	11	11	92	89	82	13	8	21	21	26	44

11.4 Discussion

In the Riverland and Barossa Valley trials, reducing the irrigation level significantly increased LWP in both years of the trial, except in the case of Shiraz on rootstock 110 Richter. This indicated increased water stress, given that grapevines are considered to be in water deficit at 10 Bar (Hellman 2010), but decreased the susceptibility of canes to colonisation by *E. lata*. This is contrary to previous reports based on incidence of infection of pruning wounds, where less irrigation led to greater incidence (Sosnowski *et al*. 2011; SAR 06/01). This suggests that the plant defence mechanisms in response to wound infection may be different from those that are involved in resisting fungal growth through vascular tissue. During vine pruning, it was observed that fully irrigated vines were most vigorous and had the thickest canes and, as the irrigation treatments were reduced, so too was the cane width. In

an Honours study, Hamblin (2015) reported that tolerant cultivars, in which *E. lata* colonisation was restricted, generally had narrower canes and xylem vessels than susceptible cultivars. This infers that narrower canes, as observed with water stressed vines in the current study, may restrict the growth of *E. lata*. Further research will be required to confirm this.

Colonisation of Cabernet Sauvignon by *Diplodia seriata* was not affected by irrigation treatments in the Riverland, but there was a significant decrease in colonisation of Shiraz, albeit less than for *E. lata*, in the Barossa Valley when vines were not irrigated.

In the Barossa Valley, there was an interaction effect between irrigation treatment and rootstocks on the susceptibility of canes to colonisation. The decrease in extent of colonisation of Shiraz canes on non-irrigated vines compared with irrigated vines was most prominent on rootstocks 110 Richter and 140 Ruggeri for *E. lata* and on rootstocks,99 Richter, Ramsey and 110 Richter as well as own rooted vines for *D. seriata*. There was no impact of irrigation on susceptibility of Shiraz canes on rootstocks; 1103 Paulsen, Schwarzmann and 99 Richter as well as own-rooted vines for *E. lata*, and rootstock: 1103 Paulsen and 140 Ruggeri for *D. seriata*. Conversely, there was an increase in susceptibility to *E. lata* colonisation for Shiraz canes on Ramsey rootstock in non-irrigated vines. For the same trial site, Kidman *et al.* (2015) reported the lowest yields for cv. Shiraz on 1103 Paulsen, and the highest on Ramsey and 110 Richter rootstocks, when vines were not irrigated. There does not appear to be a link between yield performance and susceptibility to colonisation by *E. lata*.

As was reported for cultivar and rootstock susceptibility in Section 10, there appears to be no link between the pathogens in terms of potential tolerance of the grapevine to either trunk disease, and site-specific recommendations may be necessary, depending on the predominant pathogen species in that vineyard.

In these trials on both Cabernet Sauvignon and Shiraz cultivars, *E. lata* colonised canes to a significantly greater extent than *D. seriata*, providing further data to those reported in the cultivar susceptibility trials in Section 10. This contradicts previous reports based on extent of staining that *D. seriata* had pathogenicity similar to *E. lata* (Pitt *et al*. 2013). As was observed for both pathogens in these field trials and those in Section 10, and that previously reported for *E. lata* (Sosnowski *et al.* 2007b), there was no correlation between staining and pathogen recovery. In the present study, the pathogen was recovered up to 198 mm (*E. lata*) and 177 mm (*D. seriata*) ahead of staining, compared with 80 mm in the previous study involving *E. lata* in several different cultivars (Sosnowski *et al.* 2007b), which suggests that the current recommendation of cutting at least 10 cm beyond any stained wood during remedial surgery may be insufficient to ensure the fungus is removed. Also, as shown in the present work and in Section 10, both pathogens, in individual instances, can colonise to a distance of more than 200 mm below the wound surface over 12 months, which is further than the 115 mm previously reported for *E. lata* (Sosnowski *et al.* 2007b). Therefore, further research is required to assess a greater distance along canes.

Overall, in both trials for both pathogens, utilising different cultivars in different locations, the incidence of infection and colonisation of canes was generally greater in 2013-14 than in 2014-15, coinciding with higher temperatures and rainfall in the 14 days following pruning and inoculation. This concurs with reports of optimal conditions for growth of *E. lata* (Carter 1991) and *D. seriata* (Úrbez-Torres 2011). Natural infection, as recovered from non-inoculated canes, was greatest in 2014-15 at both locations, corresponding to greater rainfall in the two days prior to and post pruning, with almost no rainfall recorded in the same period in 2013-14 in either location. It is widely reported that spores of both pathogens are released from infected wood during rainfall, can persist for a number of days and wounds are most susceptible immediately after pruning, thereafter decreasing exponentially in susceptibility to infection (Carter 1991; Úrbez-Torres 2011).

In conclusion, water deficit did not increase the susceptibility of grapevine canes to infection of pruning wounds and colonisation of the subtending tissue by trunk disease pathogens and, in fact, there was evidence of decreased susceptibility to colonisation in vines under severe water deficit. These results suggest that drought and deficit irrigation practices are not likely to contribute to an increased prevalence of grapevine trunk disease in vineyards.

12 OUTCOMES AND RECOMMENDATIONS

12.1 Objective 1: Ascertain inoculum dispersal throughout the pruning season

Outputs:
Determine climatic conditions required for spore release by ED and BD pathogens and inoculum dispersal throughout the growing season and, in particular, the pruning season, in different climatic regions of Australia.

Performance targets:
- Deploy and develop spore trap protocols
- Develop PCR primers and protocols for spore trap tape
- Quantify inoculum levels over time during season
- Correlate inoculum levels to climatic conditions

Burkard spore traps were deployed in four wine regions of Australia with differing climates, and protocols were developed for logistics, maintenance, collection and storage of spore tapes. Rapid and highly sensitive DNA-based molecular tools were designed and optimised so that multi-species primers could detect and quantify ED and BD pathogens from the environment, in particular from spore tapes. Analysis of samples has provided crucial knowledge on the dispersal of inoculum in four Australian wine regions, and its association with seasonal climatic changes. This study has provided a foundation on which to expand our understanding of the effects of environmental factors on spore dispersal, to enable the provision of recommendations on the timing of pruning and wound protection.

Previous studies on spore dispersal patterns relied on conventional techniques including microscopy and culturing on agar plates, that are extremely time consuming, less accurate and less sensitive than molecular tools. The use of quantitative DNA-based molecular tools has become popular for the detection and quantification of plant pathogens and to our knowledge, this is the first time that qPCR methods have been developed to target multiple species of ED and BD pathogens from spore trap samples. A DNA extraction protocol, based on the Gentra® Puregene® (Qiagen) system was developed and validated to give consistent yields of DNA, and was safe and easy to use. Multi-species primers developed for ED and BD were validated for specificity to the nine species that have been associated with each disease in Australia.

A limitation with these molecular tools is that they cannot discriminate to the species level, but a DNA macroarray is currently being developed at the Summerland Research and Development Centre in Canada that can detect many different GTD pathogen species from a single sample (spore tape or grapevine tissue). Adoption of this technology would provide greater knowledge on the prevalence of specific species of GTD pathogens in Australian vineyards and nurseries, to facilitate the development of more specific recommendations for disease management. However, this would require investment in specialised equipment that is currently unavailable. Another area of improvement would be the ability to undertake the qDNA analysis in the field, and in subsequent research (SAR 1601) the use of loop-mediated isothermal amplification (LAMP) assays will be investigated, which could eventually lead to a real-time spore alert system.

To date, the analysis of only one year of spore tapes has been completed from each site: Barossa Valley, Coonawarra, Hunter Valley and Griffith, due to time constraints, but analysis will continue until data from all available spore tapes are generated. Preliminary results showed that BD and ED spores were released at different times of the year, but the seasonal release and number of spores differed between pathogens and regions. ED spores were generally detected in greater numbers than BD spores, possibly due to the size and mode of discharge of these spores, but, regardless, wounds can still become infected at very low spore concentrations. Rainfall was confirmed as the primary factor that triggers the release of spores, which are disseminated throughout the entire year. Therefore, wounds created as part of canopy management (i.e. sucker removal and shoot trimming) may also be at risk of infection and will need to be the focus of future research. The prevalence of rain varies seasonally between different climatic regions and therefore influences the timing of spore release. However, the reason for differences in the prevalence of pathogens between regions is yet to be fully elucidated, and will be considered once data are generated for all years of monitoring, and with additional data from more sites in the subsequent project (SAR 1601).

This study has revealed that as little as 0.2 mm of rain could initiate spore release, which is less than the previously reported 1-2 mm. Occasionally, spores were continuously trapped for up to two weeks, which also coincided with continuous rain. However, not all rain events resulted in spore release, with several periods where no spores were trapped, despite continuous rain occurring. This indicates that

fruiting structures on the wood may have been exhausted, but then regenerated after several weeks. Furthermore, spores were occasionally detected more than a week after any rainfall event. Also, spores of BD pathogens were trapped up to 20 m from vines or alternative hosts (the maximum distance of any trap in this study), significantly further than has been previously reported, prompting the need to determine how far BD spores can actually travel and the role that alternative hosts play in harbouring the pathogens. These results suggest that spore release may also be attributed to other environmental factors such as relative humidity, dew and wind speed but this will require further investigation in consultation with a climatologist.

Wine regions in Australia cover a wide area with highly diverse climates, and the comprehensive spore trapping in four major wine regions in this study provides beneficial information on the spore release patterns of ED and BD pathogens in regions with differing climates. The molecular tools developed in this study fast-tracked the analyses of the spore trap samples and allowed quantification of spores from vineyards. The data generated from samples analysed to date have demonstrated that the seasonal spore release patterns varied between pathogens and regions and that rainfall was the primary environmental factor that influenced spore release. As more data are generated from this and subsequent projects, localised recommendations will be provided for each region to assist growers in making management decisions for grapevine trunk diseases.

A manuscript for scientific publication is in preparation, and a further two or three will be prepared based on results from inoculum dispersal studies.

12.2 Objective 2: Investigate duration of pruning wound susceptibility

Outputs:
Determine the length of time for which wounds on grape cultivars are susceptible to infection by spores of ED and BD pathogens at different times during the pruning season and the link with environmental factors.

Performance targets:
- Vineyard trials to evaluate wound susceptibility over the pruning season
- Greenhouse experiments to evaluate the difference in cultivar susceptibility to pruning wound infection

Vineyard trials undertaken over three years in McLaren Vale, SA and Wagga Wagga, NSW have provided new information on the timing and duration of wound susceptibility to ED and BD pathogens in Australia. 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, 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. Overseas, variation in the susceptibility of wounds to infection reported between regions has been attributed to climatic differences, and highlights the importance of evaluating wound susceptibility in Australian regions with varying climates. Subsequent research (SAR 1601) will evaluate ED and BD pathogens in the Adelaide Hills, a region with a different climate to those used in this study.

The high recovery of *D. seriata* from non-inoculated controls across the first two pruning seasons, compared with the other pathogens in this study, indicates a high level of natural disease pressure in the vineyard, and hence the importance of wound protection to manage BD. Recovery of *N. luteum* across the pruning season was considerably lower than that of *D. seriata*, more closely reflecting results with *E. lata*. *N. luteum* was chosen to replace *D. seriata* in the NSW vineyard trials as it produces a yellow pigment in culture, allowing for rapid differentiation from *D. seriata* cultures which do not produce the pigment. *N. luteum* was not recovered from any non-inoculated control vines, eliminating the confounding issues caused by background infection when using *D. seriata*. However, these results indicate that trunk disease pathogens vary in their prevalence of inoculum and suggest that, in developing future management strategies, the most prevalent and pathogenic species should be evaluated.

Detached cane assays were also established in the greenhouse to compare wound susceptibility between six cultivars commonly grown in Australia: Shiraz, Chardonnay, Cabernet Sauvignon, Merlot, Sauvignon Blanc and Semillon. No difference in susceptibility to both ED and BD pathogens was observed, highlighting the importance of protecting pruning wounds, regardless of the cultivar grown.

This study evaluated wound susceptibility by artificial inoculation with extremely high concentrations of fungal spores, reflected in the vast difference in recovery between inoculated and non-inoculated canes. According to previous research, natural infection is likely to be in the order of 10 spores per wound, which is 50-100 times less than the concentrations used here, and reduced disease pressure has previously resulted in increased efficacy of preventative fungicide treatments. Future wound susceptibility research will utilise lower spore concentrations, more closely reflecting natural conditions.

These results suggest that, in McLaren Vale and Wagga Wagga, there may be little advantage in choosing one pruning time over another, in terms of minimising the risk of infection by the trunk disease pathogens evaluated here. However, results highlight the importance of protecting pruning wounds for at least two weeks post-pruning.

A manuscript for scientific publication will be prepared based on results from wound susceptibility studies.

12.3 Objective 3: Optimise timing of wound protection treatments

Outputs:
Evaluate the preventative and curative properties of fungicides to identify the critical time for application of wound protectants against ED and BD pathogens and seek alternative compounds that can be applied using sprayers.

Performance targets:
- Vineyard trials to evaluate critical timing for application of wound treatments
- Greenhouse experiments to evaluate alternative treatments for wound protection

Field trials were established to assess the timing of fungicide application relative to pruning, and showed that Cabrio, Emblem and Folicur exhibited curative properties when applied to pruning wounds for control of *E. lata* and *N. luteum*. Therefore a single application once a week may be sufficient to protect wounds, rather than each day following pruning activity in the vineyard. This is likely to improve logistics for grapegrowers and, together with effective application of fungicides with commercial sprayers (Sosnowski *et al.* 2013b, Ayres *et al.* 2016), will encourage greater adoption of wound protection strategies for control of grapevine trunk diseases.

All three fungicides provided preventative control of *N. luteum* for up to two weeks after application. All three fungicides controlled *E. lata* for up to a week, after which recovery from inoculated controls was significantly reduced due to natural wound healing, reducing the likelihood of infection.

Recovery of *E. lata* from the inoculated controls in the 2014 trial was very low, similar to that of the non-inoculated controls, which was most likely due to contamination with other species. This highlights a challenge of working with *E. lata*, for which inoculum can only be generated using infected grapevine wood material. Climatic conditions also influence vineyard trials, as *E. lata* spores are reliant on rainfall and are sensitive to solar radiation. These challenges can only be overcome by repetition of trials.

Recovery of *D. seriata* from inoculated controls was extremely high, which reflected disease pressure much greater than would naturally occur in the vineyard, most likely leading to the reduced efficacy of fungicide treatments. In addition, background levels of infection were high, as indicated by non-inoculated controls, also confounding results. For this reason, *N. luteum,* which produces a yellow pigment in culture, making it more readily distinguishable from *D. seriata* cultures, was used in subsequent trials. Results with *N. luteum* more closely reflected those of *E. lata*.

Considering the challenges involved with conducting field trials with *D. seriata,* and the knowledge that a number of other fungal species are involved, it may be important to evaluate pruning wound protection with other species that cause BD. Further testing of *D. seriata* (the most prevalent species in Australia) at lower spore doses will also be necessary, to evaluate the efficacy of fungicides in preventing wound infection at closer to natural inoculum levels.

As with the wound susceptibility trials, here we used very high inoculum spore concentrations that do not necessarily reflect natural disease pressure. It is therefore likely that if spore concentrations more closely reflected those that would occur naturally, the fungicides would provide greater disease control than demonstrated here.

None of the alternative products assessed was able to consistently reduce infection by either pathogen. Nonetheless, future work will continue to assess alternative products, including chitosan and vanillin, for their ability to prevent infection by trunk disease pathogens.

Results of the susceptibility trials suggest that the most important period of wound susceptibility to trunk disease pathogens is 2-3 weeks post pruning. The results of these fungicide timing trials indicate that the three fungicides assessed can control *E. lata* and *N. luteum* when wounds are treated up to six days after infection, and will continue to provide control of both pathogens for 1-2 weeks. Therefore, if applied six days post-pruning, a single application could provide up to three weeks of wound protection.

A manuscript for scientific publication is in preparation based on results from fungicide application timing studies.

12.4 Objective 4: Evaluate potential of remedial surgery to control botryosphaeria dieback

Outputs:
Remedial surgery techniques developed to manage ED-affected vines validated for efficacy on BD-affected vines.

Performance targets:
- Vineyard trials to evaluate remedial surgery

Vineyard trials were established to evaluate remedial surgery, which has previously been shown to control eutypa dieback, as a curative control strategy for grapevines with BD. Three vineyards (own-rooted and grafted) were assessed for visual symptoms, followed by cutting trunks at different heights and recording severity of cross-sectional staining in remaining stumps. Wounds were painted and then vines were monitored for water shoot production and visually assessed for disease severity.

Similar to the previous results for ED affected vines, 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, tended to produce shoots from the rootstock rather than the scion. These results suggest that remedial surgery may be more successful in own-rooted vines than grafted vines. Future research (SAR 1601) will set up new trials on grafted vines to further elucidate the effect of reworking on these vines. It will also evaluate novel methods of water shoot induction to attempt to improve the success of the technique.

In this study, the severity of dieback in untreated vines increased by 16-29% after three years of monitoring. This indicated that if infected vines were not reworked, the dieback severity would have increased by 5-10% each year, and would eventually lead to the death of vines with no intervention. In the grafted vineyard in the Hunter Valley, more than half of the treated vines were lost due to flooding, while the average severity of dieback in the untreated vines had already reached 92%. Thus, further monitoring of these vines in the new project will not continue. However, the other trials demonstrated the short-term success of remedial surgery for control of BD in own-rooted vines. Monitoring of disease recurrence for these vines will continue for at least three more growing seasons as part of project SAR 1601.

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

A scientific publication will be prepared based on results from remedial surgery studies, once more data are collected and extra trials are conducted in the new project SAR 1601.

12.5 Objective 5: Identify tolerant or resistant germplasm

Outputs:
Evaluate germplasm held by SARDI and CSIRO for response to ED and BD, to identify genetic material that can be incorporated into breeding programs to improve tolerance/resistance to trunk diseases.

Performance targets:
- Assess germplasm collection for visual symptoms of GTDs
- Greenhouse evaluation of cultivar and rootstock susceptibility to pathogen infection
- Vineyard and potted vine evaluation of cultivar susceptibility to pathogen infection

The SARDI germplasm collection located in the Barossa Valley consists of a broad range of *Vitis vinifera* cultivars and clones sourced from around the world. The collection was visually assessed for symptoms of trunk disease, identifying 41 cultivars with less than 10% disease severity, compared with up to 98% severity recorded in susceptible cultivars.

Cultivars with low disease severity were selected for evaluation of the rate of pathogen colonisation. In detached canes, seven cultivars were consistently less susceptible to *E. lata* than Shiraz and Sauvignon Blanc, with Petit Meslier and Auxerrois showing the most potential for tolerance to eutypa dieback (ED). Two cultivars, Green Veltliner and Muscadelle, were consistently less susceptible to *D. seriata*, thereby showing the most potential for tolerance to botryosphaeria dieback (BD). In the vineyard inoculated canes, Petit Verdot and Muscadelle were consistently the least susceptible to both ED and BD pathogens, indicating that they are likely to have the greatest tolerance to infection. Furthermore, Muscat a Petit Grains Rouge, Ughetta, Pinot Noir and Riesling were less susceptible than Shiraz and Sauvignon Blanc in at least one field trial for each pathogen, suggesting potential tolerance to ED and BD. Petit Meslier and Melon were consistently less susceptible to *D. seriata* than the susceptible cultivars, suggesting potential tolerance to BD only.

Results from inoculations in the vineyard were not always consistent with those in the detached cane assay. This may be due to physiological or biochemical changes in the tissues of canes once detached from the vine and the short incubation period of four weeks used for detached canes compared with 12 months in the vineyard. The results obtained here suggest that the detached cane assay may not be a reliable method for evaluating the growth rate of trunk pathogens in living vines, but this requires further investigation.

The visual assessment of trunk disease symptoms in the SARDI clonal collection indicated that the severity of trunk disease varied considerably amongst the clones of Shiraz, Cabernet Sauvignon and Chardonnay. Caution should be taken when interpreting these results as they are based on a single visual assessment and may be influenced by the effect of vine age, which differed amongst the clones in the collection. However, it provides enough evidence to warrant further investigation to determine whether certain clones might be recommended for future plantings.

A selection of CSIRO rootstock genotypes was also screened for pathogen colonisation using detached canes and varied in their susceptibility to colonisation by *E. lata* and *D. seriata*. Several genotypes of *V. vinifera* (Riesling) x *V. cinerea* genotypes were less susceptible than cv. Shiraz. Further evaluation of these and other genotypes with similar parentage is warranted and may lead to the eventual production of rootstocks with reduced susceptibility to trunk diseases. Based on results for the cultivars, it may be necessary to evaluate rootstocks further by inoculating canes on vines in the field or providing a longer incubation period in the detached canes.

There was some correlation between cane width and colonisation by ED and BD pathogens, particularly for the *V. vinifera* cultivars, which was consistent with findings from previous research. These traits may be useful markers when selecting for tolerant cultivars or new genotypes.

E. lata colonised canes to a greater extent than *D. seriata*, contradicting previous understanding that the pathogens were similar in pathogenicity. Furthermore, the fungus can grow ahead of the stained wood up to 193 mm (*E. lata*) and 94 mm (*D. seriata*) in apparently healthy wood tissue, so the previous recommendation of cutting at least 10 cm beyond any stained wood during remedial surgery may need to be increased to 20 cm to ensure the fungus is removed. However it is imperative that the distance the pathogen can occur ahead of staining in trunks is also determined. There was no correlation between staining and pathogen recovery, casting doubt over the usefulness of extent of staining as an indicator of disease severity.

Colonisation rate of *E. lata* in canes was greater than 200 mm over 12 months, which is further than the 115 mm previously reported. Further research is required to assess a greater distance along canes and determine the maximum extent of colonisation of canes in a range of cultivars and under a variety of environmental conditions over 12 months. This would provide greater confidence in pruning management strategies that remove infected tissues from vines.

A manuscript for scientific publication will be prepared based on results from disease susceptibility studies.

12.6 Objective 6: Examine the impact of drought and regulated irrigation

Outputs:
Determine the effect of stress caused by decreased water availability due to drought or regulated deficit watering practices on the susceptibility of grapevines to trunk pathogens, which will provide information for improving disease management practices.

Performance targets:
- Vineyard trials to evaluate susceptibility under water deficit irrigation

Water deficit trials established in the Riverland and Barossa Valley regions of South Australia in 2011 and 2008, respectively, provided varying levels of water stress on grapevines, indicated by increased leaf water potential, as irrigation was reduced from 100% to 0% of the standard irrigation program. Re-isolation of *E. lata* and *D. seriata* 12 months after canes were inoculated via pruning wounds revealed that increased water stress generally decreased the susceptibility of canes to colonisation. It was observed that fully irrigated vines were the most vigorous and had the thickest canes, and as the irrigation treatments were reduced, so too was the cane width. This may infer that narrower xylem vessels restrict the growth of the fungal pathogens along the cane, which has been reported for cultivars having narrow canes and xylem vessels. Further research is recommended in order to examine this hypothesis.

The susceptibility of Shiraz canes to colonisation by both pathogens decreased in non-irrigated compared with irrigated vines on rootstock 110 Richter, on 140 Ruggeri by *E. lata* and on 99 Richter, Ramsey and 110 Richter as well as on own rooted vines by *D. seriata*. Conversely, there was an increase in susceptibility to *E. lata* colonisation for Shiraz canes on Ramsey in non-irrigated vines. There was no impact of irrigation on the susceptibility of Shiraz canes on other rootstock-pathogen combinations. The interaction between rootstock and irrigation suggests rootstocks vary in their ability to cope with the soil water conditions, which may affect their response to infection.

Eutypa lata colonised canes to a significantly greater extent than *D. seriata* for both Cabernet Sauvignon and Shiraz, indicating it is the more aggressive pathogen in Riverland and Barossa Valley environments. There was no correlation between staining and pathogen recovery, confirming previous research. The pathogens were recovered up to 198 mm ahead of staining, compared with 80 mm in previous research, so the current recommendation of cutting at least 10 cm beyond any stained wood during remedial surgery may need to be increased to 20 cm to ensure the fungus is removed. However it is imperative that the distance the pathogen can occur ahead of staining in trunks is also determined. Also, both pathogens can colonise more than 200 mm below the wound surface over 12 months. Therefore, it is recommended that further research is conducted to assess a greater distance along canes to determine the maximum rate of growth, and to also determine the rate of fungal growth in older trunk wood tissue.

Overall, the incidence of infection and colonisation of canes was generally greater when temperatures and rainfall were higher in the 14 days following pruning and inoculation. Natural infection levels corresponded to higher rainfall in the two days prior to and post pruning. This corresponds with previous research which showed that spores of both pathogens are released from infected wood during rainfall, can persist for a number of days and wounds are most susceptible immediately after pruning.

In summary, water deficit did not increase the susceptibility of grapevine canes to infection of pruning wounds and colonisation of the subtending tissue by trunk disease pathogens and, in fact, there was evidence of decreased susceptibility to colonisation in vines under severe water deficit. These results suggest that drought and deficit irrigation practices are not likely to contribute to an increased prevalence of grapevine trunk disease in vineyards.

A manuscript for scientific publication will be prepared based on results from water stress studies.

13 REFERENCES

Amponsah NT, Jones EE, Ridgway HJ and Jaspers MV (2008) Production of *Botryosphaeria* species conidia using grapevine green shoots. *New Zealand Plant Protection* 61, 301-305.

Amponsah NT, Jones EE, Ridgway HJ and Jaspers MV (2009) Rain water dispersal of Botryosphaeria conidia from infected grapevines. *New Zealand Plant Protection* 62, 228–233.

Amponsah NT, Jones EE, Ridgway HJ and Jaspers MV (2014) Factors affecting *Neofusicocum luteum* infection and disease progression in grapevines. *Australasian Plant Pathology* 43, 547-556.

Anonymous (2006) *Real-Time PCR Applications Guide*. Bio-Rad Laboratories, Inc.

Ayres, M.R.; Wicks, T.J.; Scott, E.S. and Sosnowski, M.R. (2016) Developing pruning wound protection strategies for managing Eutypa dieback. *Australian Journal of Grape and Wine Research* 23, 103-111.

Ayres M, Sosnowski M and Wicks T (2011) A rapid technique for evaluating treatments for eutypa dieback control. *Wine & Viticulture Journal* 26 (6), 50-53.

Ayres M, Wicks T, Scott E and Sosnowski M (2014) Optimising pruning wound protection for the control of eutypa dieback. *Australian and New Zealand Grapegrower and Winemaker* 602, 30-33.

Baskarathevan J, Jaspers MV, Jones EE, and Ridgway HJ (2013). Development of isolate-specific markers for *Neofusicoccum parvum* and *N. luteum* and their use to study rainwater splash dispersal in the vineyard. *Plant Pathology* 62, 501-509.

Baker KF (1957) *The U.C. System for Producing Healthy Container-Grown Plants*. University of California Press, Berkeley.

Biggs AR (1987) Occurrence and location of suberin in wound reaction zones in xylem of 17 tree species. *Phytopathology* 77, 718–725.

Biggs AR (1990) Managing wound-associated diseases by understanding wound healing in the bark of woody plants. *Journal of Arboriculture* 16, 108–112.

Billones-Baaijens R, Jones EE, Ridgway HJ and Jaspers MV (2014) Susceptiblity of common rootstock and scion varieties of grapevines to Botryosphaeriaceae species. *Australasian Plant Pathology* 43, 25-31.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* 55:611–622

Butterworth SC, Jordan SA and Schilder AM (2005) Eutypa dieback: disease progress and losses in 'Concord' grapes. *Phytopathologia Mediterranea* 44, 106.

Caesar AJ and Pearson RC (1983) Environmental factors affecting survival of ascospores of *Sclerotinia sclerotiorum. Phytopathology* 73, 1024-1030.

Canny MJ and Roderick ML (2005) A second pathway for gas out of the pressure chamber – what is being squeezed? *Plant Physiology and Biochemistry* 43, 315-321

Carter MV and Moller WJ (1967) The effect of pruning time on the incidence of *Eutypa armeniacae* infection in apricot trees. *Australian Journal of Experimental Agriculture and Animal Husbandry* 7, 584-586.

Carter MV and Moller WJ (1970). Duration of susceptibility of apricot pruning wounds to infection by *Eutypa armeniacae*. *Australian Journal of Agricultural Research* 21, 915-920.

Carter MV and Moller WJ (1971) The quantity of inoculum required to infect apricot and other Prunus species with *Eutypa armeniacae*. *Australian Journal of Experimental Agriculture and Animal Husbandry* 11, 684-686.

Carter MV (1991) The status of *Eutypa lata* as a pathogen. Monograph – Phytopathological Paper No. 32. (International Mycological Institute, Surrey, UK).

Carter MV (1994) Eutypa Dieback. P. 32-34. In: Compendium of Grape Disease. R. C. Pearson and A. C. Goheen eds. APS Press, St Paul, MN.

Carter MV (1957) *Eutypa armeniacae* Hansf. and Carter, sp. nov., an airborne vascular pathogen of *Prunus armeniaca* L. in southern Australia. *Australian Journal of Botany* 5, 21-35.

Chapuis L, Richard L and Dubos B (1998) Variation in susceptibility of grapevine pruning wound to infection by *Eutypa lata* in south-western France. *Plant Pathology* 47, 463-472.

Creaser M and Wicks T (2001) Yearly variation in Eutypa dieback symptoms and the relationship to grapevine yield. *Australian and New Zealand Grapegrower and Winemaker* 452, 50-52.

Creaser M, Savocchia S, Hitch C and Wicks T (2003) Survey of the Hunter Valley and Mudgee wine regions for eutypa dieback disease. *The Australian and New Zealand Grapegrower and Winemaker* 472, 15–16.

Creaser ML and Wicks TJ (2004) Short-term effects of remedial surgery to restore productivity to *Eutypa lata* infected vines. *Phytopathologia Mediterranea* 43, 105–107.

Diguta CF, Rousseaux S, Weidmann S, Bretin S, Vincent, B, Guillox-Benatier M and Alexandre H (2010). Development of qPCR assay for specific quantification of *Botrytis cinerea* on grapes. *FEMS Microbiology Letter* 313, 81-87

Dubos B (1987) Mise au point sur les maladies de dépérissement dans le vignoble fançais. *Le Progrés Agriculture et Viticulture* 104, 135-140.

Duvivier M, Dedeurwaerder G, De Proft M, Moreau J and Legreve A (2013) Real-time PCR quantification and spatio-temporal distribution of air-borne inoculum of *Mycosphaerella graminicola* in Belgium. *European Journal of Plant Pathology* 137, 325-341

Dumot V, Menard E, Courlit Y, Ouvrie M, Desache F, Boursier N, David S, Dubos B and Larignon P (2004) Eutypa canker in the Charentes Region: results of a 10-year study on Ugni blanc. *Phytoma* 568, 4-7.

Glass NL and Donaldson GC (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied Environmental Microbiology*, 61, 1323-1330.

Elena G, Sosnowski MR, Ayres MR, Lecomte P, Benetreau C, Garcia-Figueres F and Luque J (2015) Effect of the inoculum dose of three grapevine trunk pathogens on the infection of artificially inoculated pruning wounds. *Phytopathologia Mediterranea* 54, 345-354.

Gramaje D, Ayres MR, Trouillas FP and Sosnowski MR (2012) Efficacy of fungicides on mycelial growth of diatrypaceous fungi associated with grapevine trunk disease. *Australasian Plant Pathology* 41, 295–300.

Hamblin J (2015) Factors affecting grapevine susceptibility to Eutypa dieback. Honours Thesis, University of Adelaide.

Hellman E., 2016. Irrigation scheduling of grapevines with evapotranspiration data. AgriLIFE Extension, Texas A&M University System [http://winegrapes.tamu.edu/files/2015/11/irrigationscheduling.pdf] accessed 8 December 2016.

Highet A and Wicks T (1998) The incidence of eutypa dieback in South Australian vineyards. The *Australian Grapegrower and Winemaker* Annual Technical Issue 441a, 135–136.

Kuntzmann P, Villaume S and Bertsch C (2009) Conidia dispersal of *Diplodia* species in a French vineyard. *Phytopathologia Mediterranea* 48, 150-154.

Kidman CM, Olarte Mantilla S, Dry PR, McCarthy MG and Collins C (2015) Ramsey and 110 Richter rootstocks perform well under water stress conditions in South Australia's Barossa Valley. *Wine and Viticulture Journal* 30 (1), 43-45.

Kriedemann PE and Smart RE (1971) Effects of irradiance, temperature, and leaf water potential on photosynthesis of vine leaves. *Photosynthetica* 5, 7-15.

Leavitt GM (1990) The occurrence, distribution, effects and control of *Botryodipodia theobromae* on *Vitis vinifera* in California, Arizona and northern Mexico. Ph.D. dissertation, University of California, Riverside.

Levitt J (1980) *Responses of plants to environmental stresses*. New York, USA: Academic Press.

Loschiavo A, Sosnowski M and Wicks T (2007) Incidence of eutypa dieback in the Adelaide Hills. The *Australian and New Zealand Grapegrower and Winemaker* 519, 26-29.

Luchi N, Capretti P, Surico G, Orlando C, Pazzagli M, and Pinzani P (2005). A real-Time Quantitative PCR assay for the detection of *Sphaeropsis sapinea* from inoculated *Pinus nigra* shoots. *Journal of Phytopathology* 153, 37-42.

Martin MT, Cobos R, Martin L and Lopez-Enriquez L (2012). Real-time PCR detection of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*. *Applied and Environmental Microbiology* 78, 3985-3991.

Mayorquin, JS, Wang DH, Twizeyimana M and Eskalen A (2016). Identification, Distribution, and Pathogenicity of Diatrypaceae and Botryosphaeriaceae Associated with Citrus Branch Canker in the Southern California Desert. *Plant Disease*100, 2402-2413.

McCarthy MG, Loveys BR, Dry PR and Stoll M (2002) Regulated deficit irrigation and partial rootzone drying as irrigation management techniques for grapevines. In: Deficit Irrigation Practices, Water

Reports – 22, Food and Agriculture Organisation Corporate Document Repository, Rome, Italy, 79–88, [http://www.fao.org/docrep/004/Y3655E/y3655e00.htm] accessed 15 August 2015.

Moller WJ and Carter MV (1965) Production and dispersal of ascospores in *Eutypa armeniacae*. *Australian Journal of Biological Science* 18:67-80.

Moller WJ and Kasimatis J (1980) Protection of grapevine pruning wounds from Eutypa dieback. *Plant Disease* 64, 278-280.

Moller WJ and Kasimatis AN (1981) Further evidence that *Eutypa armeniacae* - not *Phomopsis viticola* - incites dead arm symptoms on grape. *Plant Disease* 65, 429-431.

Molyneux RJ, Mahoney N, Bayman P, Wong RY, Meyer K and Irelan N (2002) Eutypa dieback in grapevines: Differential production of acetylenic phenol metabolites by strains of *Eutypa lata*. *Journal of Agriculture and Food Chemistry* 50, 1393-1399.

Munkvold GP and Marois JJ (1993) The effects of fungicides on *Eutypa lata* germination, growth and infection of grapevines. *Plant Disease* 77, 50–55.

Munkvold GP and Marois, JJ (1995). Factors associated with variation in susceptibility of grapevine pruning wounds to infection by *Eutypa lata*. *Phytopathology* 85, 249-256.

Pearson RC (1980) Discharge of ascospores of *Eutypa armeniacae* in New York. *Plant Disease* 64, 171-174.

Peros JP and Berger G (1994) A rapid method to assess the aggressiveness of *Eutypa lata* isolates and the susceptibility of grapevine cultivar to Eutypa dieback. *Agronomie* 14, 515–523.

Petzoldt CH, Moller WJ and Sall MA (1981) Eutypa dieback of grapevine: seasonal differences in infection and duration of susceptibility of pruning wounds. *Phytopathology* 71, 540-543.

Petzoldt CH, Sall MA, Moller WJ (1983) Factors determining the relative number of ascospores released by *Eutypa armeniacae* in California. *Plant Disease* 67, 857-860

Pitt WM, Huang R, Steel CC and Savocchia S (2010) Identification and distribution of Botryosphaeriaceae species associated with grapevine decline and dieback in New South Wales and South Australia. *Australian Journal of Grape and Wine Research* 16, 258-271.

Pitt WM, Sosnowski MR, Huang R, Qiu Y, Steel CC and Savocchia S (2012). Evaluation of fungicides for the management of Botryosphaeria canker of grapevines. *Plant Disease* 96, 1303-1308.

Pitt WM, Huang R, Steel CC and Savocchia S (2013). Pathogenicity and epidemiology of Botryosphaeriaceae species isolated from grapevines in Australia. *Australasian Plant Pathology* 42, 573-582.

Pouzoulet J, Mailhac N, Couderc C, Besson X, Dayde J, Lummerzheim, M and Jacques, A (2013). A method to detect and quantify *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* DNA in grapevine wood samples. *Applied Microbiology and Biotechnology* 97, 10163-10175.

Pouzoulet J, Pivovaroff AL, Santiago LS and Rolshausen PE (2014) Can vessel dimension explain tolerance toward fungal vascular wilt diseases in woody plants? Lessons from Dutch elm disease and esca disease in grapevine. *Frontiers in Plant Science* 5, 1-11.

Qiu, Y (2014). The biology and epidemiology of the grapevine trunk disease pathogen Botryosphaeriaceae spp. PhD Thesis, Charles Sturt University.

Ramos DE, Moller WJ and English H (1975) Production and dispersal of ascospores of *Eutypa armeniacae* in California. *Phytopathology* 65:1364-1371.

Ridgway HJ, Amponsah NT, Brown DS, Baskarathevan J, Jones EE and Jaspers MV (2011). Detection of botryosphaeriaceous species in environmental samples using a multi-species primer pair. *Plant Pathology* 60, 1118–1127.

Rogers SL, Atkins SD and West JS (2009) Detection and quantification of airborne inoculums for *Sclerotinia sclerotiorum* using quantitative PCR. *Plant Pathology* 58, 324-331.

Rolshausen PE, Greve LC, Labavitch JM, Mahoney NE, Molyneux RJ and Gubler WD (2008) Pathogenesis of *Eutypa lata* in grapevine: identification of virulence factors and biochemical characterization of cordon dieback. *Phytopathology* 98, 222-229.

Rotem J, Wooding B and Aylor DE (1985) The role of solar radiation, especially ultraviolet, in the mortality of fungal spores. *Phytopathology* 75, 510-514.

Savocchia S, Steel CC, Stodart BJ and Somers A (2007) Pathogenicity of *Botryosphaeria* species isolated from declining grapevines in sub-tropical regions of eastern Australia. *Vitis* 46, 27-32.

Serra S, Mannoni MA and Ligios V (2008). Studies on the susceptibility of pruning wounds to infection by fungi involved in grapevine wood diseases in Italy. *Phytopathologia Mediterranea* 47, 234–246.

Siebert JB (2001) Eutypa: The economic toll on vineyards. *Wines and Vines* April, 50–56.

Slippers B and Wingfield MJ 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Reviews* 21 90-106.

Smart RE and Coombe BG (1983) Water relations of grapevines. *In: Water deficits and plant growth*. Ed. Kozlowski TT, New York, USA: Academic Press, 137-96 (Vol. 7).

Spagnolo A, Marchi G, Peduto F, Phillips AJL, and Surico G. (2011). Detection of Botryosphaeriaceae species within grapevine woody tissues by nested PCR, with particular emphasis on the *Neofusicoccum parvum/N. ribis* complex. *European Journal of Plant Pathology* 129, 485–500.

Sosnowski MR, Shtienberg D, Creaser ML, Wicks TJ, Lardner R and Scott ES (2007a). The influence of climate on foliar symptoms of eutypa dieback in grapevines. *Phytopathology* 97, 1284-1289.

Sosnowski MR, Wicks TJ, Lardner R and Scott ES (2007b) The influence of grapevine cultivar and isolate of *Eutypa lata* on wood and foliar symptoms. *Plant Disease* 91, 924-931.

Sosnowski MR, Creaser ML, Wicks TJ, Lardner R and Scott ES (2008) Protecting grapevine wounds from infection by *Eutypa lata*. *Australian Journal of Grape and Wine Research* 14, 134-142.

Sosnowski MR, Luque J, Loschiavo AP, Martos S, Garcia-Figueres F, Wicks TW and Scott ES (2011a) Studies on the effect of water and temperature stress on grapevines inoculated with *Eutypa lata*. *Phytopathologia Mediterranea* 50, S127-S138.

Sosnowski MR, Wicks TW and Scott ES (2011b) Control of Eutypa dieback in grapevines using remedial surgery. *Phytopathologia Mediterranea* 50, S277-S284.

Sosnowski M (2012) Highlights from the international workshop on grapevine trunk diseases. The Australian and New Zealand Grapegrower and Winemaker 585, 40-42.

Sosnowski M, Ayres M, Wicks T and McCarthy M (2013a) In search of resistance to grapevine trunk diseases. *Wine & Viticulture Journal* 28(4), 55-58.

Sosnowski MR, Loschiavo AP, Wicks TJ and Scott ES (2013b) Evaluating treatments and spray application for the protection of grapevine pruning wounds from infection by *Eutypa lata*. *Plant Disease* 97, 1599-1604

Sosnowski M and Wicks T (2012) Current status of grapevine trunk diseases in Australia. *Wine & Viticulture Journal* 27, 21.

Sun Q, Rost TL and Matthews MA (2006). Pruning-induced tylose development in stems of current-year shoots of *Vitis vinifera* (Vitaceae). *American Journal of Botany* 93, 1567–1576.

Tamura K, Dudley J, Nei M and Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596-1599.

Tey-Rulh P, Philippe I, Renaud JM, Tsoupras G, De Angelis P, Fallot J and Tabacchi R (1991) Eutypine, a phytotoxin produced by *Eutypa lata* the causal agent of dying-arm disease of grapevine. *Phytochemistry* 30, 471-473.

Travadon R, Rolshausen PE, Gubler WD, Cadle-Davidson L and Baumgartner K (2013). Susceptibility of cultivated and wild *Vitis* spp. to wood infection by fungal trunk pathogens. *Plant Disease* 97, 1529-1536.

Trese AT, Burton CL and Ramsdell DC (1980). *Eutypa armeniacae* in Michigan vineyards: Ascospore production and survival, host infection, and fungal growth at low temperatures. *Phytopathology* 70, 788-793.

Trese AT, Ramsdell CD and Burton CL (1982) Effects of winter and spring pruning and post inoculation cold weather on infection of grapevine by *Eutypa armeniacae*. *Phytopathology* 72, 438-440.

Trouillas, FP (2009) Taxonomy and biology of Eutypa and other diatrypaceae species associated with grapevine canker diseases in California. Ph.D. dissertation, University of California, Davis.

Trouillas FP, Pitt WM, Sosnowski MR, Huang R, Peduto F, Loschiavo A, Savocchia S, Scott ES and Gubler WD (2011). Taxonomy and DNA phylogeny of Diatrypaceae associated with *Vitis vinifera* and other woody plants in Australia. *Fungal Diversity* 49, 203–223.

Úrbez-Torres JR, Battany M, Bettiga LJ, Gispert C, McGourty G, Roncoroni J, Smith RJ, Verdegaal, P and Gubler WD (2010) Botryosphaeriaceae species spore-trapping studies in California vineyards. *Plant Disease* 94, 717-724.

Úrbez-Torres JR and Gubler WD (2011) Susceptibility of grapevine pruning wounds to infection by *Lasiodiplodia theobromae* and *Neofusicoccum parvum*. *Plant Pathology* 60, 261–270.

Úrbez-Torres JR (2011). The status of Botryosphaeriaceae species infecting grapevines. *Phytopathologia Mediterranea* 50, S5–S45.

Van de Wouw AP, Stonard JF, Howlett, BJ, West JS, Fitt, BDL, and Atikins SD (2010). Determining frequencies of avirulent alleles in airborne *Leptosphaeria maculans* inoculum using quantitative PCR. *Plant Pathology* 59, 809-818.

Valencia D, Torres C, Camps R, Lopez E, Celis-Diez J and Beosain X (2015) Dissemination of Botryosphaeriaceae conidia in vineyards in the semiarid Mediterranean climate of the Valparaíso Region of Chile. *Phytopathologia Mediterranea*. 54, 394-402.

van Niekerk JM, Calitz FJ, Halleen F and Fourie PH (2010) Temporal spore dispersal patterns of grapevine trunk pathogens in South Africa. *European Journal of Plant Pathology* 127, 375-390

van Niekerk JM, Calitz FJ, Halleen F, Fourie PH (2011a) Temporal susceptibility of grapevine pruning wounds to trunk pathogen infection in South African grapevines. *Phytopathologia Mediterranea* 50, 139-150.

van Niekerk J, Strever A, Du Toit G, Halleen F, Fourie P (2011b) Influence of water stress on Botryosphaeriaceae disease expression in grapevines. *Phytopathologia Mediterranea* 50, 151–165.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninski, & T. J. White (Eds.), *PCR protocols: A guide to methods and applications* (pp. 315–322). San Diego: Academic Press.

Wicks T (1975) The dying arm disorder of vines in South Australia. *Agricultural Record* 2, 14-20.

Wicks T and Davies K (1999) The effect of Eutypa on grapevine yield. *Australian Grapegrower and Winemaker* 426a, 15-16.

Wunderlich N, Ash G, Steel C, Raman H and Savocchia S (2011) Association of Botryosphaeriaceae grapevine trunk disease fungi with the reproductive structures of *Vitis vinifera*. *Vitis* 50, 89-96.

Zhao Z, Liu H, Luo Y, Zhou S, An L, Wang C, Jin Q, Zhou M and Xu J (2014). Molecular evolution and functional divergence of tubulin superfamily in the fungal tree of life. Scientific Reports 4 : 6746

14 COMMUNICATION

14.1 Publications

14.1.1 Scientific publications

It is anticipated that a further eight scientific publications will be produced from results generated in this project with two manuscripts already in preparation.

Ayres, M.R.; Wicks, T.J.; Scott, E.S. and Sosnowski, M.R. (2016) Developing pruning wound protection strategies for managing Eutypa dieback. *Australian Journal of Grape and Wine Research* 23, 103–111.

Gramaje D, Baumgartner K, Halleen F, Mostert, L, Sosnowski MR, Úrbez-Torres JR and Armengol J (2015) Fungal trunk diseases: a problem beyond grapevines? *Plant Pathology* 65, 355-356.

Elena G, Sosnowski MR, Ayres MR, Lecomte P, Benetreau C, Garcia-Figueres F and Luque J (2015). Effect of the inoculum dose of three grapevine trunk pathogens on infection of artificially inoculated pruning wounds. *Phytopathologia Meditteranea* 54, 345-354.

Pitt WM, Huang R, Steel CC, Savocchia S (2013). Pathogenicity and epidemiology of Botryosphaeriaceae species isolated from grapevines in Australia. *Australasian Plant Pathology* 42, 573-582.

Sosnowski MR, Loschiavo AP, Wicks TJ and Scott ES (2013) Evaluating treatments and spray application for the protection of grapevine pruning wounds from infection by *Eutypa lata*. *Plant Disease* 97, 1599-1604.

Pitt WM, Trouillas FP, Gubler WD, Savocchia S and Sosnowski MR (2013) Pathogenicity of diatrypaceous fungi on grapevines in Australia. *Plant Disease* 97, 749-756.

14.1.2 Conference papers

Sosnowski M, Ayres M, Billones-Baaijens R, Savocchia S, Wicks T and Scott E (2016) Innovations in the management of grapevine trunk diseases. Keynote presentation abstract - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 43.

Savocchia S, Billones-Baaijens R, Ayres M and Sosnowski M (2016) Management of botryosphaeria dieback in grapevines using remedial surgery. Poster 116 - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 76.

Billones-Baaijens R, Ayres M, Sosnowski M and Savocchia S (2016) Spore-trapping studies for trunk disease pathogens in Australian vineyards. Poster 117 - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 76.

Ayres M, Billones-Baaijens R, Savocchia S, Scott E and Sosnowski M (2016) Critical timing for application of pruning wound protectants for control of grapevine trunk diseases. Poster 121 - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 77.

Sosnowski M, Ayres M, Wicks T, McCarthy M and Scott E (2016) Investigating potential for resistance to grapevine trunk disease. Poster 122 - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 77.

Ayres M, Billones-Baaijens R, Savocchia S, Scott E and Sosnowski M (2016) Susceptibility of pruning wounds to grapevine trunk diseases. Poster 123 - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 77.

Sosnowski M, Ayres M and Scott E (2016) The influence of water deficit on grapevine trunk disease progression. Poster 124 - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 77.

Hamblin J, Sosnowski M, Burton R and Scott E (2016) The influence of cane morphology and composition on susceptibility to eutypa dieback. Poster 129 - *The 16th Australian Wine Industry Technical Conference*, Adelaide Convention Centre, SA, 24-28 July 2016. P 77.

Sosnowski M and Úrbez Torres J (2016) Grapevine trunk diseases – a new emergence of old diseases. In proceedings of the International Cool Climate Symposium, Brighton, England 26-28 May 2016 P.24.

Sosnowski M (2015) Sustaining vineyards through management of grapevine trunk diseases. In proceedings of ASVO Seminar: Vineyard Longevity, Maintaining the Asset. Mildura Arts Centre, 22-23 July 2015, P. 9.

Sosnowski MR, Ayres MR, Wicks TJ, McCarthy M and Scott ES (2015) Investigating potential resistance of grapevines to eutypa and botryosphaeria dieback. Proceedings of the *European Cooperation in Science and Technology (COST) Action FA 1303 Workshop - Sustainable control of grapevine trunk diseases*, Cognac, France, 23-24 June 2015.

Sosnowski MR, Ayres MR, Wicks TJ and Scott ES (2015) Optimising pruning wound protection for control of eutypa dieback. Proceedings of the *European Cooperation in Science and Technology (COST) Action FA 1303 Workshop - Sustainable control of grapevine trunk diseases*, Cognac, France, 23-24 June 2015.

Sosnowski MR, Ayres MR, Wicks TJ, McCarthy M and Scott ES (2014). Searching for resistance to grapevine trunk diseases. Proceedings of the 9th International Workshop on Grapevine Trunk Diseases, Adelaide Australia 18-20 November, p 43.

Ayres MR, Wicks TJ, Scott ES and Sosnowski MR (2014). Developing effective pruning wound protection against eutypa dieback in grapevine. Proceedings of the 9th International Workshop on Grapevine Trunk Diseases, Adelaide Australia 18-20 November, p 48.

Elena G, Benetreau, Lecomte P, Ayres MR, Sosnowski MR and Luque J (2014). Optimizing techniques for evaluating Eutypa lata infection in grapevines. Proceedings of the 9th International Workshop on Grapevine Trunk Diseases, Adelaide Australia 18-20 November, p 62.

Ayres MR, Mundy DC, Wicks TJ, Scott ES and Sosnowski MR (2014). A detached cane assay is an effective tool for evaluating pruning wound infection. Proceedings of the 9th International Workshop on Grapevine Trunk Diseases, Adelaide Australia 18-20 November, p 63.

Savocchia S, Ayres MR, Billones-Baaijens and Sosnowski MR (2014). Remedial surgery for the management of Botryosphaeria dieback in grapevines. Proceedings of the 9th International Workshop on Grapevine Trunk Diseases, Adelaide Australia 18-20 November, p 72.

Sosnowski MR, Mundy DC and Belayneh T (2014). Evaluation of Gelseal for control of eutypa dieback. Proceedings of the 9th International Workshop on Grapevine Trunk Diseases, Adelaide Australia 18-20 November, p 73.

Sosnowski M (2014) Identification and management of trunk diseases in Australia. Proceedings of the 7th International Table Grape Symposium, Mildura, Australia 12-14 November, p 59.

Ayres M, Wicks T, Scott, E and Sosnowski M (2013) Optimising pruning wound protection for management of eutypa dieback in grapevine. *19th Australasian Plant Pathology Conference*, Auckland, NZ, 25-28 November, p 67.

Sosnowski M, Ayres M, Wicks T and McCarthy M (2013) In search of resistance to grapevine trunk diseases. *19th Australasian Plant Pathology Conference*, Auckland, NZ, 25-28 November, p 94.

Pitt WM, Trouillas FP, Gubler WD, Savocchia S and Sosnowski MR (2013) Pathogenicity of species implicated in the Eutypa dieback disease of grapevines. *19th Australasian Plant Pathology Conference*, Auckland, NZ, 25-28 November, p 119.

Sosnowski M. (2013) Wood diseases – a silent assassin. *19th Annual Romeo Bragato Conference*, Blenheim, New Zealand, 29 August 2013, p.76-77.

Sosnowski M. (2013) Managing grapevine trunk disease. *19th Annual Romeo Bragato Conference*, Blenheim, New Zealand, 29 August 2013, p.82-83.

Pitt WM, Huang R, Steel CC, Savocchia S (2013). Pathogenicity of Botryosphaeriaceous fungi isolated from grapevines in southeastern Australia. *15th Australian Wine Industry Technical Conference (AWITC)*, Sydney, Australia, 13–18 July.

14.1.3 Book chapter

Rolshausen P, Sosnowski M, Trouillas FP and Gubler WD (2015) Eutypa dieback in Compendium of Grape Diseases, Disorders and Pests (2nd Ed) Eds. WF Wilcox, WD Gubler and JK Uyemoto. Pp. 57-61.

14.1.4 Industry journals and articles

Ayres M, Billones-Baaijens R, Savocchia S, Scott E and Sosnowski M (2016) Susceptibility of pruning wounds to grapevine trunk disease pathogens. *Wine and Viticulture Journal* 31(6), 48-50.

Sosnowski M, Ayres M, Wicks T, McCarthy M and Scott E (2016) Investigating potential for resistance to grapevine trunk diseases. *Wine and Viticulture Journal* 31(5), 41-45.

Sosnowski M, Ayres M and Scott E (2016) The influence of water deficit on grapevine trunk disease. *Wine and Viticulture Journal* 31(4), 46-50.

Anonymous (2016) The battle against Eutypa. *Australian and New Zealand Grapegrower and Winemaker* 628, 48-52.

Anonymous (2016) There's more to trunk disease than meets the eye. Wine Australia R&D News, May 2016. [http://research.wineaustralia.com/theres-more-to-trunk-disease-than-meets-the-eye/]

Jermyn I (2015) Vignerons hear it on the grapevine. Central Western Daily, Orange NSW, 23 Oct 2015, Pg 8.

Savocchia S and Billones-Baaijens R (2015) Trunk diseases: back to basics with plant pathology. *Wine & Viticulture Journal* 30(3): 48-50.

Sosnowski M (2015) Highlights from the 9th International Workshop on Grapevine Trunk Diseases. *Wine and Viticulture Journal* 30(3): 51-53.

Anonymous (2015) Eutypa breakthrough in time for pruning season/Pruning expo. *Australian and New Zealand Grapegrower and Winemaker* 616, 54-58.

Anonymous (2015) Death knell for eutypa dieback. *Rural Business* Feb 2015, p. 30.

Anonymous (2014) Scientists unravel grapevine mystery. *South East Coastal Leader* 10 December 2014, p 12.

Anonymous (2014) Global focus on grapevine disease management. 'Today's top story' Daily Wine News (online), 24 November 2014.

Sampson A (2014) International Conference – Hope on grapevine killer. *Weekly Times (Victoria)* 26 November 2014, p 73.

Carne N (2014) Finding an answer to trunk disease in grapevines. *The Lead, South Australia*, 11 November 2014 [http://www.theleadsouthaustralia.com.au/industries/primary-industries/finding-an-answer-to-trunk-disease-in-grapevines/]

Anonymous (2014) Barossa stages its first pruning expo. *Stock Journal*. 14 August 2014, p15.

Anonymous (2014) Australian first with grapevine trunk disease workshop. *Australian and New Zealand Grapegrower and Winemaker* 607, 40.

Anonymous (2014) Save our vines. *Wine Business Magazine*, August 2014 p 36.

Opray M (2014) Dieback dilemma. *Stock Journal* 17 July 2014 p 3.

Anonymous (2014) Helping control Eutypa. *Leader (Barossa Valley)* 25 June 2014 p 47.

Fowler, S (2014) Eutypa dieback research study is ongoing. *Herald (Barossa Valley)* 25 June 2014 p 2.

Anonymous (2014) Sprayable pesticide shows promise against vine disease. *Countryman (WA) 3 April 2014, p 18.*

Anonymous (2014) Eutypa - a hot topic in the Barossa. *Leader (Barossa Valley)* 16 April 2014, p 22.

Anonymous (2014) Relief for wine grape growers. *Weekly Times (Victoria)* 9 April 2014, p 79.

Ayres M, Wicks T, Scott E and Sosnowski M (2014) Optimising pruning wound protection for the control of eutypa dieback. *Australian and New Zealand Grapegrower and Winemaker* 602, 30-33.

Anonymous (2014) Fungicide arsenal boosts eutypa dieback control. *Stock Journal* 20 March 2014, p 8.

Anonymous (2014) Research breakthrough in fight to control eutypa disease. GWRDC Innovators Network e-Newsletter. January 2014.

Waite H (2013). Understanding trunk diseases: How and why they threaten the wine industry. *Wine and Viticulture Journal*, November/December. (With input from Sosnowski and Savocchia. H Waite represents the trunk diseases action group of which Sosnowski and Savocchia are members).

Sosnowski M and Ayres M (2013) SARDI Grapevine trunk disease research trial. *The Grower's Voice*, McLaren Vale Grape, Wine and Tourism, Jul 2013, p 6.

Sosnowski M, Ayres M, Wicks T and McCarthy M (2013) In search of resistance to grapevine trunk diseases. *Wine & Viticulture Journal* 28(4), 55-58.

Anonymous (2013) Practical management options for controlling trunk disease in grapevines. GWRDC Innovators Network e-Newsletter. July 2013. http://www.gwrdc.com.au/trunk-disease-in-grapevines/.

Barwick J (2013). Growers respond to trunk disease threat. GWRDC Innovators Network e-Newsletter. June 2013. http://www.gwrdc.com.au/growers-respond-to-trunk-disease-threat.

Gannaway K (2013) Forum tackles threats. *Mountain Views Mail*. 7 May 2013, p. 5.

Anonymous (2013) Trunk disease workshop delivers latest knowledge. *The Australian and New Zealand Grapegrower and Winemaker*, 593, 13.

Pitt WM, Huang R, Steel CC, Savocchia S (2013) Pathogenicity of fungi causing Botryosphaeria dieback of grapevines in Australia. *Wine and Viticulture Journal*, 28(4), 59–61.

Pitt WM, Savocchia, S (2013) Symptoms and management of Bot canker. *The Grape Press*, 158, 17–19.

Jones L (2013) Research aims for eutypa dieback fungicide registration. Australian and New Zealand Grapegrower and Winemaker 591, GWRDC newsletter R&D at work p2.

Anonymous (2013) Eutypa dieback research targeting fungicide registration. GWRDC Innovators Network e-Newsletter. March 2013. http://www.gwrdc.com.au/eutypa-dieback-research-targeting-fungicide-registration/

14.2 Presentations & radio/television interviews

Billones-Baaijens, R. and Savocchia, S. (2016). Practical Management of Grapevine Trunk Diseases. Recent research on major grape diseases and new disease resistant grape varieties workshop. 16 November 2016. (30 attendees), National Wine and Grape Industry Centre, Charles Sturt University.

Sosnowski M and Ayres M (2016) Eutypa dieback survey and assessment training days. Wine Australia SA Central Regional Program. McLaren Vale Visitor Centre 25 Oct (15 attendees), CMV Vineyards, Langhorne Creek 27 Oct (10 attendees), Nepenthe Vineyards Adelaide Hills 15 Nov (7 attendees).

Sosnowski M (2016) Practical management of grapevine trunk disease. Accolade Wines Viticultural Meeting, AWRI Boardroom, Urrbrae, SA. 7 Sep (12 attendees)

Sosnowski M (2016) Eutypa dieback – best practice management guide. Clare Region Wine and Grape Association, Eutypa extension launch, Rising Sun Hotel, Auburn, SA on 5 Sep 2016 (40 attendees).

Sosnowski M (2016) Practical management of grapevine trunk disease. Roberts Viticulture Day, The Grange, Campbell Town, Tas. 4 August (115 attendees)

Sosnowski M, Ayres M, Billones-Baaijens R, Savocchia S, Wicks T and Scott E (2016) Innovations in the management of grapevine trunk diseases. Keynote presentation - The 16th Australian Wine Industry Technical Conference, Adelaide Convention Centre, SA, 24-28 July 2016. (1,100 delegates)

Sosnowski M (2016) Managing trunk disease in the Vineyard. Workshop 31: Future-proofing your viticultural Enterprise. The 16th Australian Wine Industry Technical Conference, Adelaide Convention Centre, SA, 24-28 July 2016. (30 attendees)

Sosnowski M (2016) Practical management of eutypa dieback. Farmer Johns Viticulture Seminar, Peter Lehmann Wines, Tanunda, SA. 6 July (90 attendees)

Baaijens RB (2016) Grapevine trunk disease problems down under. Summerland Research and Development Centre, Agriculture and Agri-Food Canada, Summerland, BC, Canada, 25 May 2016 (50 attendees).

Savocchia S, Billones-Baaijens R, Ayres M, Sosnowski M (2016) Trapping with views to kill. Airborne Pest & Disease Surveillance Workshop, Adelaide, 17 – 18 May 2016 (30 attendees).

Sosnowski M (2016) Eutypa dieback – best practice management guide. Limestone Coast Grape and Wine Council, Eutypa BPMG launch, Coonawarra Hall, SA 16 June 2016 (40 attendees).

Sosnowski M (2016) Practical management of grapevine trunk diseases. Managing Trunk Diseases – Omnia Grower Workshop, RACV Healesville Country Club, Vic. 9 June 2016 (20 attendees)

Sosnowski M (2016) Practical management of grapevine trunk diseases. VBH Supplies and Crop Care winter viticulture update, Langhorne Creek, SA 3 June 2016 (12 attendees).

Sosnowski M and Urbez Torres J (2016) Grapevine trunk diseases – a new emergence of old diseases. Session: Emerging vineyard pests and diseases, International Cool Climate Symposium, Brighton, England 26-28 May 2016 (132 attendees).

Sosnowski M (2016) Grapevine trunk diseases and Management. Grapevine disease workshop, South West Vineyard Association, Aldwick Court Farm and Vineyard, Redhill, England, 23 May 2016 (13 attendees).

Sosnowski M (2016) Recent insights into the management of eutypa dieback in grapevines. Victorian Trunk Disease Management Workshops, Mornington Peninsula, North East and Western Victoria, 26-28 April 2016 (82 attendees)

Sosnowski M (2016) Management of grapevine trunk diseases. SA Central Trunk Disease Workshops, Adelaide Hills, 19 April (80 attendees) and McLaren Vale, 22 April 2016 (40 attendees).

Ayres M (2016) Practical management of grapevine trunk diseases – progress update. SA Central Trunk Disease Workshops, Adelaide Hills, 19 April (80 attendees), and McLaren Vale, 22 April 2016 (40 attendees).

Sosnowski M (2016) SARDI Grapevine trunk disease research. Victoria Horticulture Industry Network meeting, Adelaide, 5 April 2016 (20 attendees)

Kidman C, Sosnowski M, DeGaris K (2015) Eutypa impacts: ground trothing infra-red technologies. Crush 2015 – the grape and wine science symposium, Plant Research Centre, Urrbrae SA, 20 Nov 2015 (128 attendees)

Ayres M, Sosnowski M, Wicks T and Scott E (2015) Developing strategies for management of eutypa dieback. Crush 2015 – the grape and wine science symposium, Plant Research Centre, Urrbrae SA, 20 Nov 2015 (128 attendees)

Sosnowski M and Ayres M (2015) Management of eutypa dieback in grapevines, Practical management of grapevine trunk diseases. Limestone Coast Grape and Wine Council – Grapevine trunk disease workshop. Coonawarra Hall, SA, 17 Nov 2015. (27 attendees)

Sosnowski M and Ayres M (2015) Management of eutypa dieback in grapevines, Practical management of grapevine trunk diseases. Clare Region Winegrape Growers Association – Grapevine trunk disease workshop and Disease assessment training. Annie's Lane Winery, Watervale and vineyard near Auburn, 2-4 Nov 2015. (33 attendees)

Savocchia S (2015) Practical management of grapevine trunk diseases. NSW Wine Industry Innovation Forum, Orange, NSW 2-3 Nov.

Sosnowski M (2015) Management of eutypa dieback in grapevines. NSW Spring Vine Health Workshops. Orange, Murrumbateman and Tumbarumba, 22-24 October 2015 (52 attendees).

Sosnowski M and Billones-Baaijens R (2015) Practical management of grapevine trunk diseases 2013-2016. NSW Spring Vine Health Workshops. Orange, Murrumbateman and Tumbarumba, 22-24 October 2015 (52 attendees)

Billones-Baaijens R (2015) Botryosphaeria dieback - symptoms, epidemiology and management. NSW Spring Vine Health Workshops. Orange and Murrumbateman, 22-23 October.

Savocchia S (2015) Botryosphaeria dieback - symptoms, epidemiology and management. NSW Spring Vine Health Workshops. Tumbarumba, 24 October.

Sosnowski M (2015) Television interview – Grapevine Trunk Diseases in the Central West of NSW. Aired 6.14 pm on 21 October 2015 on Prime 7 Orange (2.03 mins)

Savocchia S (2015) Botryosphaeria dieback - symptoms, epidemiology and management. NSW Spring Vine Health Workshops. Griffith, Mudgee, Hunter Valley, 9-11 September.

Savocchia S (2015) Management of eutypa dieback in grapevines. NSW Spring Vine Health Workshops. Griffith, Mudgee, Hunter Valley, 9-11 September.

Billones-Baaijens R (2015) Practical management of grapevine trunk diseases 2013-2016. NSW Spring Vine Health Workshops. Griffith, Mudgee, Hunter Valley, 9-11 September.

Savocchia S (2015) Television interview – Grapevine trunk diseases. Aired on WIN News in Wagga Wagga, Orange and Albury. 10 Sept 2015. (total audience coverage 92, 909).

News articles in The Northern Star, Lismore; The Daily Examiner; The Weekly, Mudgee NSW; The Rural, Wagga Wagga NSW; Irrigator, Leeton, NSW; Area News, Griffith, NSW; eg. http://www.northernstar.com.au/news/local-growers-get-latest-vineyard-update/2759270/

Sosnowski M (2015) Eutypa update. Muirs Information Session, Parri Estate, McLaren Flat, 26 August 2015 (30 attendees).

Sosnowski M (2015) Management of grapevine trunk disease. Aglink Vitilink Meeting, Mercure Hotel, Mildura, 23 July 2015 (13 attendees).

Savocchia S (2015) Grapevine trunk diseases. Lecture and practical class delivered to undergraduate students at Charles Sturt University, Wagga Wagga NSW, 27 June 2015. (33 attendees)

Sosnowski (2015) Controlling trunk diseases at pruning time. 2015 Barossa Pruning Expo, Nuriootpa Research Centre 3 June (170 attendees)

Sosnowski (2015) Controlling trunk diseases at pruning time. Landmark webinar, 4 June (18 attendees)

Sosnowski (2015) Management of eutypa dieback in grapevines & Practical management of grapevine trunk diseases – progress update. Treasury Wine Estates Trunk Disease Workshops: Magill Estate 21 April (20 attendees), Heathcote Vineyard, Victoria 23 April (15 attendees); Coonawarra Vineyard 30 April (16 attendees).

Sosnowski M (2015) Current status of grapevine trunk disease in Australia. Phylloxera and Grape Industry Board of South Australia board meeting, National Wine Centre, Adelaide 2 February 2015.

Sosnowski M and Billones-Baaijens R (2014) Practical management of grapevine trunk diseases 2013-2016. Wine Victoria Grapevine Trunk Disease Workshop Series. Yarra Valley, Mornington Peninsula, Geelong, Wangaratta, Bendigo and Ararat, 8-10 & 15-17 December 2014 (156 attendees).

Savocchia S (2014) Botryosphaeria dieback – Symptoms, Epidemiology and Management. Wine Victoria Grapevine Trunk Disease Workshop Series. Yarra Valley, Mornington Peninsula, Geelong, Wangaratta, Bendigo and Ararat, 8-10 & 15-17 December 2014 (156 attendees).

Sosnowski M (2014) Management of eutypa dieback in grapevines. Wine Victoria Grapevine Trunk Disease Workshop Series. Yarra Valley, Mornington Peninsula, Geelong, Wangaratta, Bendigo and Ararat, 8-10 & 15-17 December 2014 (156 attendees).

Sosnowski M (2014) 9th International Workshop on Grapevine Trunk Diseases in Adelaide & Breakthrough in the management of grapevine trunk diseases. Radio interview; ABC North & West SA, Riverland SA and South East SA, 18/20 November 2014.

Sosnowski M (2014) Identification and management of trunk diseases in Australia. Keynote address at the 7th International Table Grape Symposium, Mildura, Australia 12-14 November (300 attendees).

Sosnowski M (2014) Keynote presentation at the 7th International Table Grape Symposium. TV interview; WIN News Mildura 13 November 2014.

Ayres M (2014) How is research helping grape growers control trunk disease? Webinar, Australian Wine & Research Institute, 6 November. (16 attendees)

Sosnowski M (2014) Management of Eutypa and Botryosphaeria dieback in grapevines; NZ Wine project update – Practical management of grapevine trunk diseases. SBS Bank Rural Grape Vine

Trunk Disease seminar, Central Otago Grapegrowers Association, Cromwell, New Zealand, 13 August 2014 (21 attendees)

Sosnowski M (2014) Managing Grapevine Trunk Diseases in Australia and New Zealand. 14th Annual Enology & Viticulture Conference of the British Columbia Wine Grape Council, Penticton, BC, Canada, 21-22 July 2014 (250 attendees)

Sosnowski M (2014) Grapevine trunk disease research. Radio interview; ABC North & West SA, Riverland SA and South East SA. 22 July 2014

Savocchia S (2014) Identification and management of grapevine trunk diseases. Lecture and practical delivered to undergraduate students at Charles Sturt University, Wagga Wagga NSW, 26 June 2014. (21 attendees)

Sosnowski M (2014) Techniques to identify, prevent and manage Eutypa. Barossa Pruning Expo. Barossa Grape and Wine Association. Nuriootpa Research Centre, 11 June 2014. (80 attendees)

Sosnowski M (2014) Managing eutypa dieback in grapevines – practical strategies and research update. Clare Region Winegrape Growers Association Workshop, Rising Sun Hotel, Auburn, SA, 6 June 2014. (30 attendees)

Sosnowski M (2014) Grapevine trunk disease research update. Elders Viticulture Growers Information Evening, Tanunda Football Club, Barossa Valley, 4 June 2014. (110 attendees)

Sosnowski M (2014) Grapevine trunk disease research update. GWRDC Regional program reporting meeting. Hickinbotham Roseworthy Wine Science Laboratory, Urrbrae, 28 May 2014. (15 attendees)

Sosnowski M (2014) Management of grapevine trunk disease. Muirs annual viticulture meeting. Rendezvous Restaurant, Mildura, 6 May 2014. (25 attendees)

Sosnowski M (2014) Management of eutypa dieback in grapevines. Barossa Valley Estate Grower Workshop, Barossa Valley Estate Cellar Door, 1 May 2014. (20 attendees)

Sosnowski M (2014) Management of eutypa dieback in grapevines. Barossa Grape & Wine Association Eutypa dieback Workshop, Tanunda Football Club, 23 April 2014. (70 attendees)

Ayres M (2014) Eutypa dieback project update. Barossa Grape & Wine Association Eutypa dieback Workshop, Tanunda Football Club, 23 April 2014. (70 attendees)

Sosnowski M (2014) Managing eutypa dieback of grapevines. Tasmanian Grapevine Trunk Disease Workshops, Stoney Vineyard Coal River Valley 11 February 2014 (5 attendees) and Jansz Vineyard, Tamar Valley 12 February 2014. (12 attendees)

Savocchia S (2014) Symptoms, pathogenicity and management of Botryosphaeria dieback. Queensland Wine Industry Association Grapevine Trunk Disease Workshops, Stoney Vineyard Coal River Valley 11 February 2014 (5 attendees) and Jansz Vineyard, Tamar Valley 12 February 2014. (12 attendees)

Sosnowski M (2014) Limestone Coast Eutypa Survey Results and 8th International Workshop on Grapevine Trunk Diseases. Pre-Vintage Breakfast Seminar, Coonawarra Grape and Wine Incorporated, Coonawarra Hall, 7 February 2014. (65 attendees)

Ayres M (2014) Optimising management of eutypa dieback. Pre-Vintage Breakfast Seminar, Coonawarra Grape and Wine Incorporated, Coonawarra Hall, 7 February 2014. (65 attendees)

Sosnowski M (2014) Optimising management of eutypa dieback. Radio interview ABC North & West SA. 16 January 2014

Sosnowski M (2013) Detection of eutypa dieback in grapevines. Workshop 6: Detection of diseases of woody tissue, 19th Australasian Plant Pathology Conference in Auckland, NZ, 29 November 2013.

Sosnowski M (2013) Management of eutypa dieback in grapevines. Queensland Wine Industry Association Grapevine Trunk Disease Workshops, Ballandean Estate, Ballandean Qld, 6 November 2013 (30 attendees), Clovely Estate, Moffatdale, Qld, 8 November 2013 (18 attendees).

Ayres M (2013) Managing eutypa dieback – practical solutions for growers. Queensland Wine Industry Association Grapevine Trunk Disease Workshops, Ballandean Estate, Ballandean Qld, 6 November 2013 (30 attendees), Clovely Estate, Moffatdale, Qld, 8 November 2013 (18 attendees).

Savocchia S (2013) Symptoms, pathogenicity and management of Botryosphaeria dieback. Queensland Wine Industry Association Grapevine Trunk Disease Workshops, Ballandean Estate,

Ballandean Qld, 6 November 2013 (30 attendees), Clovely Estate, Moffatdale, Qld, 8 November 2013 (18 attendees).

Sosnowski M (2013) Grapevine trunk disease research briefing. GWRDC Regional Program Planning Day. Nuriootpa Research Centre, Barossa Valley, 31 October.

Sosnowski M (2013) Management of eutypa dieback in grapevines. Eutypa Management Day. Adelaide Hills Wine Region and GWRDC Regional. Nepenthe Cellar Door, Balhannah, 23 October (41 attendees).

Ayres M & Sosnowski M (2013) Managing eutypa dieback – practical solutions for grape growers. McLaren Vale Grape, Wine and Tourism grower information session, McLaren Vale & Fleurieu Visitor Information Centre, 25 September 2013. (25 attendees)

Sosnowski M (2013) Managing eutypa dieback in grapevines. Webinar, Australian Wine & Research Institute, 17 September. (36 attendees)

Savocchia S (2013) Identification and management of grapevine trunk diseases. Lecture and practical delivered to undergraduate and Masters students at Charles Sturt University, Wagga Wagga NSW, 7 September 2013 (12 attendees).

Pitt WM (2013) 1. Identifying Botryosphaeria dieback – symptoms and epidemiology and 2. Managing Botryosphaeria dieback in mature grapevines. Workshop 44: Trunk diseases – practical steps for managing an insidious threat to vine health and productivity. *15th Australian Wine Industry Technical Conference (AWITC)*, Sydney, Australia, 18 July. (20 attendees)

Sosnowski MR (2013) 1. Identifying eutypa dieback - symptoms and epidemiology and 2. Managing eutypa dieback in mature grapevines. Workshop 44: Trunk diseases – practical steps for managing an insidious threat to vine health and productivity. *15th Australian Wine Industry Technical Conference (AWITC)*, Sydney, Australia, 18 July. (20 attendees)

Sosnowski M (2013) Eutypa dieback of grapevines. Elders Viticulture Growers Information Evening, Tanunda Football Club, Barossa Valley, SA 5 June 2013. (85 attendees)

Pitt W (2013) Symptoms, pathogenicity and management of Botryosphaeria dieback. Victorian Viticultural Association workshop. Healesville Memorial Hall, Yarra Valley, Victoria 23 April 2013. (69 attendees)

Sosnowski M (2013) Eutypa dieback of grapevines, Victorian Viticultural Association workshop. Healesville Memorial Hall, Yarra Valley, Victoria 23 April 2013. (69 attendees)

15 STAFF

Principal investigator: Mark Sosnowski

Chief Investigators: Sandra Savocchia
Matthew Ayres
Regina Billones-Baaijens

Advisors: Eileen Scott, Chris Steel, Gavin Ash and Trevor Wicks

SARDI technical assistance: Lee Bartlett, Ian Bogisch, Cathy Todd

NWGIC technical assistance: David Foster, Lindsay Greer, Jennifer Bannister, Meifang Liu, Peter Carey, Gurli Nielsen

16 ACKNOWLEDGEMENTS

We acknowledge input from Wayne Pitt into project development and establishment of field trials before his resignation early in the project.

We would like to thank the following:
Regional grower associations that provided generous financial support, vineyard resources and technical input into this project; particularly Adelaide Hills Wine Region, Barossa Grape and Wine Association, McLaren Vale Grape, Wine and Tourism Association, Clare Region Winegrape Growers Association, Limestone Coast Grape and Wine Council, Coonawarra Grape and Wine Incorporated, Treasury Wine Estates, Riverina Wine Grapes Marketing Board, McWilliam's Wines, Hunter Valley Wine and Tourism Association.

Nufarm/Cropcare for financial support and providing products for evaluation trials.
Bayer CropScience for providing products for evaluation trials.

Rohan Kimber (SARDI) for advice and support with spore trapping methodology.

José Úrbez Torres (Agri-Canada) for advice and supervision of Reggie Billones-Baaijens during the development and validation of molecular tools at the Summerland Research and Development Centre in Canada.