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Diagnosis and management of eutypa dieback



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GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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TABLE OF CONTENTS

1 Abstract 4

2 Executive summary 5

3 Background 7

4 Project Aims 10

5 Methods 12

5.1 Epidemiology 12
5.1.1 Ascospore inoculum 12
5.1.1.1 Ejection of ascospores 12
5.1.1.2 Effect of rainfall on ascospore release and germination 12
5.1.1.3 Interaction between distance of ascospore ejection and temperature 12
5.1.2 Growth of *E. lata* in grapevine wood 12
5.1.2.1 Extent of infection in grapevine 12
5.1.2.2 Effect of cultivar on growth rate of E. lata 12
5.1.3 Foliar symptoms 13
5.1.3.1 Relationship between foliar symptoms and yield 13
5.1.3.2 Seasonal variation of foliar symptoms 14
5.1.4 Pathogenicity of *E. lata* and cultivar susceptibility 14
5.1.5 Survey of Hunter Valley and Mudgee, New South Wales 16

5.2 Prevention 17
5.2.1 Evaluation of treatments for the prevention of wound infection 17
5.2.1.1 Laboratory evaluation 17
5.2.1.2 Field evaluation 18
5.2.2 The effect of wound size on efficacy of wound treatments 21
5.2.3 The efficacy of spray application of wound treatments 23
5.2.4 Residue analysis 23
5.2.4.1 Benlate (a.i. benomyl) 23
5.2.4.2 Garrison (a.i. cyproconazole + iodocarb) 24

5.3 Control 25
5.3.1 Remedial surgery 25
5.3.2 Grafting 26
5.3.3 Injection trials 26
5.3.4 Brotomax trials 26
5.3.5 *Trichoderma* trials 27

5.4 Diagnosis of eutypa dieback 28
5.4.1 Development of DNA markers specific to *E. lata* 28
5.4.1.1 Development of SCAR markers for a PCR–based assay 28
5.4.1.2 Assessment of SCAR and ITS markers developed in France and California 28
5.4.1.3 Development of markers for a Southern hybridisation–based assay 28
5.4.2 Detection of *E. lata* in grapevine wood using DNA markers 28
5.4.2.1 PCR–based detection of E. lata 28
5.4.2.2 Southern hybridisation–based detection of E. lata 29
5.4.3 Identification of *E. lata* from mycelium using SCAR markers 29
5.4.4 Genetic variation in *E. lata* 29
5.4.4.1 RAPD analysis 29
5.4.4.2 RFLP analysis 29
5.4.5 Production of phenolic secondary metabolites by *E. lata* 30
5.4.5.1 Identification and characterisation of a specific chemical marker 31
5.4.5.2 Identification of chemical marker(s) in treated and infected vines 32

6 Results and Discussion 34

6.1 Epidemiology 34
6.1.1 Ascospore inoculum 34
6.1.1.1 Ejection of ascospores *34*
6.1.1.2 Effect of rainfall on ascospore release and germination *34*
6.1.1.3 Interaction between distance of ascospore ejection and temperature *34*
6.1.2 Growth of *E. lata* in grapevine wood 35
6.1.2.1 Extent of infection in grapevine *35*
6.1.2.2 Effect of cultivar on growth rate of E. lata *36*
6.1.3 Foliar symptoms 37
6.1.3.1 Relationship between foliar symptoms and yield *37*
6.1.3.2 Seasonal variation of foliar symptoms *38*
6.1.4 Pathogenicity of *E. lata* and cultivar susceptibility 40
6.1.5 Survey of Hunter Valley and Mudgee, New South Wales 47

6.2 Prevention 49
6.2.1 Evaluation of treatments for the prevention of wound infection 49
6.2.1.1 Laboratory evaluation *49*
6.2.1.2 Field evaluation *50*
6.2.2 The effect of wound size on efficacy of wound treatments 57
6.2.3 The efficacy of spray application of wound treatments 58
6.2.4 Residue analysis 60
6.2.4.1 Benlate (a.i. benomyl) *60*
6.2.4.2 Garrison (a.i. cyproconazole) *60*

6.3 Control 61
6.3.1 Remedial surgery 61
6.3.2 Grafting 64
6.3.3 Injection trials 64
6.3.4 Brotomax trials 64
6.3.5 *Trichoderma* trials 65

6.4 Diagnosis of eutypa dieback 66
6.4.1 Development of DNA markers specific to *E. lata* 66
6.4.1.1 Development of SCAR markers for a PCR–based assay *66*
6.4.1.2 Assessment of SCAR and ITS markers developed in France and California *66*
6.4.1.3 Development of markers for a Southern hybridisation–based assay *66*
6.4.2 Detection of *E. lata* in grapevine wood using DNA markers 67
6.4.2.1 PCR–based detection of E. lata *67*
6.4.2.2 Southern hybridisation–based detection of E. lata *67*
6.4.3 Identification of *E. lata* from mycelium using SCAR markers 68
6.4.4 Genetic *variation* in *E. lata* 69
6.4.4.1 RAPD analysis *69*
6.4.4.2 RFLP analysis *69*
6.4.5 Production of phenolic secondary metabolites by *E. lata* 71
6.4.5.1 Identification of culture media and preliminary assessment of secondary metabolite production by E. lata *71*
6.4.5.2 Identification and characterisation of a specific chemical marker *72*
6.4.5.3 Identification of chemical marker(s) in treated and infected vines *80*

7 Outcomes and conclusions 85
7.1 Epidemiology 85
7.2 Prevention 86
7.3 Control 87
7.4 Diagnosis 88
8 Recommendations 90
8.1 Epidemiology 90
8.2 Prevention 90
8.3 Control 91
8.4 Diagnosis 91
Appendix 1: Communication 92
Appendix 1.1. Communication within the project 92
Appendix 1.2. Communication with project stakeholders (including CRCV, GWRDC, R&D agencies and industry reference groups) 92
Appendix 1.3. Communication with industry and scientific audiences 92
Appendix 1.4. Publications 92
Appendix 1.5. Presentations 95
Appendix 1.6. Refereed scientific journal articles published during the project 99
Appendix 2: Intellectual property 100
Appendix 3: References 101
Appendix 4: Staff 105
Appendix 5: Acknowledgements 106
Appendix 6: Other relevant material 107
Appendix 7: Budget reconciliation 109

1 ABSTRACT

The objectives of this project were to study the epidemiology, management and diagnosis of eutypa dieback to develop integrated management strategies for industry. Growth rate of *Eutypa lata* varied significantly among cultivars. Foliar symptoms of eutypa dieback varied annually and were not a reliable indicator of infection. A strong correlation was observed between symptom severity and yield loss. Analysis of climate data indicated that rainfall and temperature influenced severity and incidence of foliar symptoms. Numerous treatments were evaluated as pruning wound protectants to prevent eutypa dieback. Bavistin was the most suitable replacement for Benlate, which has been withdrawn from the market. Preliminary studies showed that application using commercial spray equipment may be as effective as applying with a paint brush. Acrylic paints or pastes, with or without fungicides, were also effective wound protectants. Remedial surgery to eliminate *E. lata* from infected vines was effective in the short–term, however, further research is required to assess the long–term success of this technique. Rapid identification and detection methods for *E. lata* were developed using polymerase chain reaction and Southern hybridisation techniques to generate specific DNA markers. These markers were used to detect the pathogen in infected wood. Specific biochemical markers for *E. lata* were identified, however, these could not be detected in the foliage of infected vines.

2 EXECUTIVE SUMMARY

Eutypa dieback is a degenerative disease of mature vines which threatens the sustainability of premium Australian vineyards. Vines become infected when ascospores of the fungus *Eutypa lata* infect wounds made in the wood. The fungus grows slowly in the wood and produces foliar symptoms several years after infection. At the start of this project, no fungicides or biological agents were registered for the control of eutypa dieback in Australia, however, Vinevax (*Trichoderma* spp.) has since been registered. Remedial surgery is often undertaken to restore vines to productivity. There is a need to improve strategies for the management of eutypa dieback, based on a sound understanding of epidemiology. Also, rapid diagnostic techniques will facilitate studies of epidemiology and control, and improve the prospects for management of this intractable disease.

Methods for studying the release of ascospores from infected, dead wood in the laboratory were developed. Spores were ejected up to 10 mm above the surface of dead wood. Release of spores from wood was greater for Grenache than that of Shiraz. Ascospore release increased with increasing volume of simulated rainfall applied, from 1 to 20 mL, but was not affected by increasing the temperature from 4 to 22°C. Growers are recommended to avoid pruning or reworking during or immediately following rainfall >1 mm to reduce the potential for infection.

Foliar symptoms in naturally infected, mature Shiraz vines varied from year to year, and there was a constant relationship between the severity of foliar symptoms and yield. Variation in rainfall and temperature was related to the incidence of foliar symptoms and further work is required to confirm if these factors influence disease development. Foliar symptoms were induced on young grapevine cuttings within 8 months of inoculation with mycelium in a shadehouse. This was considerably faster than that in mature field vines, which did not express foliar symptoms 4 years after inoculation. The absence of foliar symptoms was not a reliable indicator that a vine was healthy, as shown by the presence of discoloured wood in vines examined during re–working trials. Discoloured wood, which contained the pathogen, was observed in below–ground parts of the trunk, but not in the roots. Such vines would not be candidates for reworking. *E. lata* grew at up to 50 mm/yr in mature Cabernet Sauvignon and Shiraz vines in the field, while in the shadehouse a maximum rate of 115 mm/yr was recorded on Grenache rootlings. The staining of wood typically associated with eutypa dieback was not correlated with the spread of the fungus, and *E. lata* was isolated up to 80 mm in advance of staining in young vines. Growth rate of *E. lata* and foliar symptoms of the disease varied significantly among cultivars.

A survey of the Hunter Valley and Mudgee regions of New South Wales showed that eutypa dieback was not responsible for the dead arms prevalent on mature vines, as isolation yielded *Botryosphaeria* spp. which cause the disease known as Bot canker or Black dead arm. These trunk diseases may be associated with the warm to hot, sub–tropical climate of the Hunter Valley whereas the Mediterranean climate of southern Australia is more suited to eutypa dieback. This distribution reflects research in California.

The benzimidazole fungicides Benlate (benomyl) and Bavistin (carbendazim) were the most effective of the materials evaluated for protection of pruning wounds from infection following artificial inoculation. Application of Benlate to pruning wounds did not result in residues in the fruit. Because Benlate and related products have been withdrawn from the market, steps have been taken to extend the label registration of Bavistin for use on grapevine wounds to control eutypa dieback. Other fungicides, such as Cabrio (pyraclostrobin), BASF 516 (boscalid + pyraclostrobin), Nustar (flusilazole), Shirlan (fluazinam), Scala (pyrimethanil) and Fungaflor (imazalil) were effective but require further evaluation to obtain registration for this purpose. Application of fungicides using commercial spray equipment would reduce the time involved in application and reduce the cost of protecting pruning wounds. Preliminary trials showed that

spray application can be as effective as application with a paint brush, but further research is required to develop an efficient method suitable for adoption by growers.

None of the biological products tested was effective. However, other reports from Australia and abroad claim that *Trichoderma* spp. (as Trichoseal or Vinevax) applied to wounds reduces infection by *E. lata* and Vinevax is now registered for this use in Australia. Further testing is necessary to determine the reasons for the variable control. Physical barriers such as paints and pastes were as effective as fungicides. Acrylic paint prevented infection by *E. lata* and paste products such as Garrison and Nectec, which contain fungicides, were also effective. Steps are being taken to register Garrison for this purpose.

Remedial surgery to restore eutypa dieback–affected vines to productivity is a labour intensive and costly process. In the short–term, remedial surgery is an effective strategy to manage affected vines. The success of this method is affected by cultivar and the extent of infection. Growers are advised to monitor vines in early spring for foliar symptoms and to tag symptomatic cordons for removal by remedial surgery in winter and that, when removing stained tissue, growers should cut back a further 10 cm or more to ensure removal of *E. lata* mycelium in advance of the staining. Finally, wounds need to be protected by application of acrylic paint, paste or fungicide. Long–term evaluation is required to confirm the benefits of remedial surgery.

The injection of fungicides or *Trichoderma* spp. into the trunks of infected vines had no influence on grape yield or symptoms of eutypa dieback. Foliar application of Brotomax increased yield of infected vines, only after 3 years of treatment, but with no reduction in disease symptoms. This approach is not recommended for the control of eutypa dieback in grapevine.

DNA markers for use in PCR (polymerase chain reaction) and Southern hybridisation assays were developed to identify *E. lata* in culture and detect the fungus in infected grapevine wood. Once validated, the PCR assay was used routinely throughout the project to confirm the identity of isolates suspected to be *E. lata*. Only the Bio-101 soil DNA extraction kit yielded DNA of a quality suitable for PCR amplification without inhibition by the phenolic compounds which occur in discoloured, diseased wood. However, this kit costs approximately A$1,000 for 50 DNA extractions, and is not cost–effective for use in routine detection of *E. lata* DNA. Southern hybridisation, a technique much less susceptible to inhibition by phenolic compounds, allowed the reliable detection of *E. lata* DNA in artificially inoculated and naturally infected wood. Detection of *E. lata* DNA in mature wood and annual canes using Southern hybridisation was more sensitive than re–isolation into culture and provided an accurate and efficient means of assessing the efficacy of potential control agents.

Analysis of secondary metabolites produced by *E. lata* showed that the production of potentially phytotoxic acetylenic secondary metabolites was most consistent in culture media containing grapevine wood and sucrose. Eutypine, previously implicated as the toxin responsible for the foliar symptoms of eutypa dieback, was not produced by all isolates. The related compound, eutypinol, was produced by all isolates except one and was selected for use as a biochemical marker to detect the pathogen in infected vines. Eutypinol was detected in pure cultures of the pathogen using HPLC (high performance liquid chromatography), TLC (thin layer chromatography) and UV spectroscopy. HPLC analysis also identified eutypinol in micropropagated grapevines inoculated with mycelium of *E. lata*. However, neither eutypinol nor any other metabolites of *E. lata* could be detected in grapevines naturally infected with *E. lata* using HPLC analysis, TLC or Raman spectroscopy. Phytotoxicity tests showed that extracts from cultures of *E. lata* were toxic to grapevine leaf discs and indicated that compounds other than acetylenic phenols may contribute to the foliar symptoms characteristic of eutypa dieback. *In vitro* studies showed that secondary metabolite production by *E. lata* is strongly influenced by temperature and water availability, which may have implications for managing vines infected with the pathogen.

3 BACKGROUND

Eutypa dieback, caused by the fungus *Eutypa lata*, is a major disease of grapevines in Australia and worldwide. Infected grapevines gradually decline and eventually die. Surveys have shown that eutypa dieback is widespread in many of the premium winegrowing areas of Australia (Wicks, 1975; Highet and Wicks, 1998). In 1999 the disease was estimated to cause yield losses of at least 860 and 740 kg/ha on Shiraz and Cabernet Sauvignon, respectively, costing $A1,000–3,000 per hectare (Wicks and Davies, 1999). The cost to the Californian grapevine industry was estimated at US$260 million per annum (Siebert, 2001). Premium red varieties, such as Cabernet Sauvignon and Shiraz, appear to be most severely affected and in some vineyards more than 40% of vines show symptoms of eutypa dieback (Highet and Wicks, 1998). In South Australia, over 8% of mature Shiraz vines are infected with *E. lata* and these vines can be expected to die within 10 years. Similar levels of infection can be expected in grape growing areas in other states where conditions are conducive for this disease.

A degenerative disease of mature vines, eutypa dieback is often associated with vines reworked to change the cultivar or to adapt the vine to a different trellising system or for mechanical harvesting. Since many vineyards in Australia are undertaking such practices, losses to the disease may increase in the future. In addition, the problem may be exacerbated in regions where vineyards co–exist with orchards. Fruit trees such as apricot and apple are common alternative hosts for *E. lata* (Carter, 1991), and dead or diseased branches provide inoculum for initiating disease in nearby vineyards. Other factors, including grape cultivar, cultural practice and climate, are likely to influence the incidence, severity and spread of the disease.

E. lata infects when ascospores (sexual spores) land on pruning wounds and the fungus then grows slowly in the vascular tissue of the cordons and trunk of the vine. After many years, the fungus produces a canker and internal staining (Figure 3.1), blocking the transport of water and nutrients to upper parts of the vine. Foliar symptoms, comprising stunted shoots with chlorotic leaves, often cupped and with tattered margins, are thought to be caused by toxic metabolites produced by the fungus in the wood and transported to the foliage (Moller and Kasimatis, 1981; Mauro *et al.*, 1988; Tey–Rulh *et al.*, 1991; Figure 3.2). Delayed manifestation of foliar symptoms makes the disease difficult to detect until extensive invasion has occurred, by which time drastic and expensive remedial measures must be undertaken to prevent further spread of the disease.

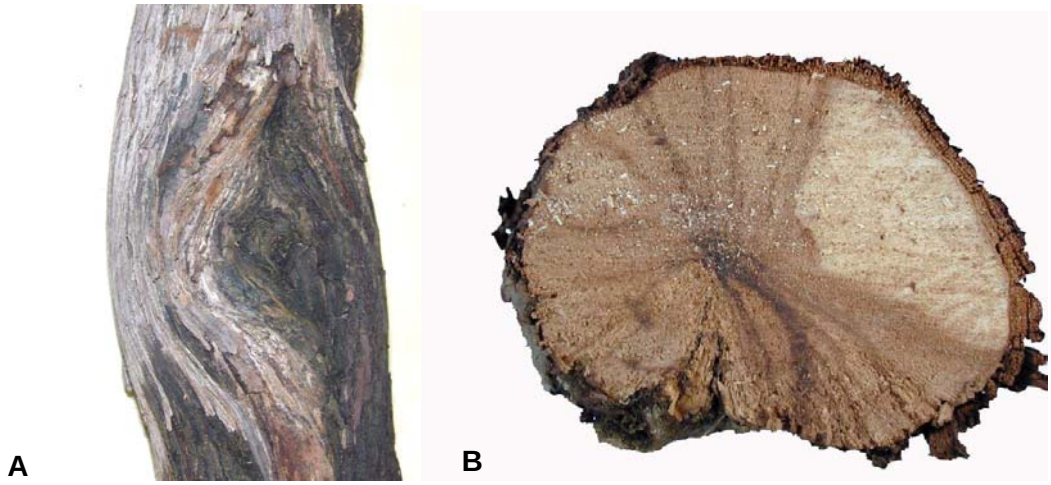


Figure 3.1. Wood symptoms caused by *E. lata*, showing (A) the external canker and (B) the wedge of internal staining.



Figure 3.2. Infected vine showing characteristic foliar symptoms of eutypa dieback.

There are few data on the epidemiology or control of eutypa dieback in grapevines in Australia, since most of the available information is based on research on eutypa dieback of apricots (see Carter, 1991). Pioneering work on eutypa dieback was conducted at the Waite Institute in South Australia by Maurice Carter. Much of the research concerned apricots and it was not until the 1970s that it was revealed that the fungus also attacked grapevines (Carter, 1975). The temperature and rainfall in a district influence the amount and timing of ascospore production. Perithecia (fruiting structures) and ascospores are produced on wood in regions where there is at least 350 mm of annual rainfall (Moller and Carter, 1965; Carter 1991). Ascospore release from diseased grapevine wood has been reported to occur after approximately 1–2 mm of rainfall (Moller and Carter 1965, Emmett and Hamilton 1994). However, information on the effects of temperature and rainfall on ascospore release is based largely on observation and controlled experiments are lacking. Investigation of the conditions in which spores are produced, liberated and dispersed, and the conditions in which infection may or may not occur will provide a basis for developing cultural and chemical controls.

Preventing infection that results from colonisation of cut surfaces by ascospores is likely to be the most effective long–term approach to controlling eutypa dieback. Research on chemical and biological control as well as the period of susceptibility of wounds to infection by *E. lata* has been undertaken in California. Pruning wounds are frequently treated with a benzimidazole fungicide, such as Benlate or Spin, both of which have been evaluated extensively in Australia on apricot but not grapevine (Carter, 1991). In experiments conducted overseas, a number of chemical and biological treatments protected pruning wounds from infection by *E. lata* (Carter, 1991; Munkvold and Marois, 1993). However, these treatments had not been evaluated for the control of eutypa dieback on grapevine in Australia and no chemical or biological agent was registered in Australia at the start of this project. Benlate® was taken off the market in 2001.

The efficacy of fungicidal chemicals for the control of spore germination, infection and progression of *E. lata* through vine tissue was investigated in laboratory and glasshouse studies. Fungicides, potential biocontrol organisms and wound protectants were tested for their efficacy in preventing infection by airborne ascospores. Fungicides and potential biocontrol organisms were tested in field trials for the ability to retard the progression of infection and development of symptoms. In addition, the influence of different pruning techniques, training and reworking practices on infectivity was studied. This will allow the development of recommendations for minimising the susceptibility of large wounds through pruning timing and technique.

Currently, it is difficult to evaluate control procedures (such as the application of chemicals or biological wound colonisers) in the field as results are not evident until 3–4 years after treatment. The development of rapid procedures for detecting *E. lata* will facilitate research and indexing of mature vines. DNA probes for identifying *E. lata* and detecting the fungus in mature wood of trunks and cordons will be useful as a research tool for evaluating control strategies. DNA primers specific to Australian isolates of the pathogen were developed for use in PCR–based assays and a DNA probe for use in Southern hybridisation assays was developed to detect the pathogen in infected wood. PCR–based assays for the detection of *E. lata* in grapevine wood have also been developed in France (Lecomte *et al.*, 2000) and California (Irelan *et al.*, 1999); these protocols were evaluated in this project.

Although DNA–based identification is sensitive, it relies on sampling host tissue that contains mycelium of *E. lata*, and false negative results are possible if samples are taken from healthy tissue within an infected vine. In contrast, it is assumed that metabolites of *E. lata* are distributed throughout the sap and foliage of infected vines, in particular in the spring time when foliar symptoms are most evident. Hence, diagnosis based on identifying specific metabolites of *E. lata in planta* may enable reliable detection of the pathogen before it spreads extensively throughout the vine. High performance liquid chromatography (HPLC) was used to identify toxins and other metabolites produced by *E. lata* that would be suitable for use in a diagnostic test for the pathogen. Biochemical and spectroscopic assays, including HPLC and Raman spectroscopy, were tested for the ability to detect such metabolites in the foliage of infected vines, as a non–destructive means of diagnosis and indexing of mature vines.

Research on eutypa dieback in the CRCV began in 1999 as part of project 2.2.1 “Managing grapevine trunk diseases (Petri disease, esca, eutypa dieback and others) that threaten the sustainability of Australian viticulture”. Research from 1999–2000 was reported in the Interim Final Report submitted to the CRCV and GWRDC in September 2003 (Pascoe *et al.*, 2003). Research on eutypa dieback from 2003–06 was conducted as part of the CRCV supplementary funding bid as CRCV S2.2.4 “Diagnosis and management of eutypa dieback”.

4 PROJECT AIMS

Aims:

To develop:

- Field management strategies that are compatible with practices for growing quality wine–grapes and which can be recommended to owners of *Eutypa*–affected and unaffected vineyards.
- Enhanced understanding of the interactions between the vine, pathogen and environment and therefore increased knowledge of the epidemiology of eutypa dieback.
- A rapid means of diagnosis of eutypa dieback, based on the identification and detection of chemical markers in vine foliage by high performance liquid chromatography (HPLC), liquid chromatography mass spectrometry (LCMS) and/or near infra–red reflectance spectroscopy (NIR).

Output 1: Epidemiological studies of *E. lata.*
Performance Targets:
- Growth rate of *E. lata* quantified in selected grapevine cultivars by isolation and DNA–based techniques.
- Symptom development compared in selected grapevine cultivars.
- Underlying cause of annual variation in symptom expression determined, if possible.
- The rate of disease progress over consecutive years determined to identify when remedial action should be taken and/or when vineyards should be replanted.

Output 2: Develop biological and chemical control strategies for eutypa dieback.
Performance Targets:
- Wound protection treatments identified that can be applied effectively using commercial spray equipment within several days of pruning, that provide long–lasting protection, and that may also have curative properties.
- The efficacy of wound protection treatments applied to large wounds, i.e. wounds greater than 20 mm in diameter, established.
- The efficacy of wound protection treatments applied in 2001 to 20–year–old Sauvignon Blanc vines when top–worked to Shiraz assessed.

Output 3: Evaluate curative/fungistatic treatments to restore productivity to *Eutypa*–infected vines.
Performance Targets:
- Efficacy of novel products for the treatment of infected vines assessed.
- The most effective method of conducting remedial surgery determined.
- The short– and long–term effects of remedial surgery in removing infected tissue and restoring vineyard productivity and longevity determined.

Output 4: Information on chemical marker(s) for use as a diagnostic marker for *E. lata*.
Performance Targets:
- Identification and characterisation, by HPLC and LCMS, of unique chemical marker(s) consistently produced by *E. lata* in pure culture.
- Evaluation of NIR as a means of rapid detection of chemical marker(s) in extracts of pure cultures of *E. lata*.

Output 5: Information on detection of chemical marker(s) in extracts or ground foliage of treated and inoculated vines.

Performance Targets:

- Evaluation of HPLC, LCMS and/or NIR for detection of chemical marker(s) in foliage of vines injected with the chemical marker(s).
- Evaluation of HPLC, LCMS and/or NIR for detection of chemical marker(s) in foliage of vines inoculated with *E. lata* in tissue culture and in the glasshouse.

Output 6: Information on detection of chemical marker(s) in foliage of naturally infected vines.

Performance Targets:

- Evaluation of HPLC, LCMS and/or NIR for detection of chemical marker(s) in foliage of naturally infected vines.
- Comparison of spectra for foliar extracts and whole leaves, using lab–based NIR and hand–held radiometer.

Output 7: Validation of chemical marker(s) as an indicator of infection using DNA–based probe.

Performance Target:

- Application of DNA–based marker to confirm the presence of *E. lata* in naturally infected vines.

Output 8: Information on response of vines to chemical and biological treatments.

Performance Target:

- Use chemical markers and spectroscopic techniques to monitor the response of vines to treatment with chemical or biological control agents identified above.

5 METHODS

5.1 Epidemiology

5.1.1 Ascospore inoculum

Ascospore release was examined by Linda–Sue Ward, an undergraduate student at the University of Adelaide. Dead wood or old prunings of Grenache and Shiraz with blackened surfaces characteristic of infection by *E. lata* were collected from the ground in a vineyard known to be affected by eutypa dieback in the Southern Vales of South Australia in winter 2001. Infection by *E. lata* was confirmed by examination of perithecia and ascospores, and by culturing on agar medium.

5.1.1.1 Ejection of ascospores

A protocol was developed to study release of ascospores from stromata. Small chips of stromata–covered wood were placed in water at approximately 22ºC for 1 hour then surface sterilised in ethanol. These wood chips were transferred to Petri dishes containing a thin layer of potato dextrose agar (PDA) in both the base and the lid, so that the approximate distance between the wood and the agar in the lid was either 5 or 10 mm. The Petri dishes were incubated the right way up at approximately 22ºC for 2 weeks, and the number of wood samples which ejected ascospores to a distance of 5 or 10 mm was determined by counting the number of colonies of *E. lata* growing on the agar in the lid.

5.1.1.2 Effect of rainfall on ascospore release and germination

Wood chips with stromata were prepared as above, and sprayed with water to simulate rainfall. Using a rain gauge and a hand–held spray bottle, a fine mist was sprayed over the wood chips until the required volume was reached (0, 1, 2 or 20 mL). The wood chips were air–dried in the sun (ambient temperature of 22ºC) and placed 5 mm below a layer of PDA as described above.

5.1.1.3 Interaction between distance of ascospore ejection and temperature

Wood chips with stromata were prepared as above and then transferred to PDA plates so that distances of 2, 4, 6, 8 and 10 mm were achieved between the wood chip and agar surface. Plates were incubated at either 5 or 22ºC.

5.1.2 Growth of *E. lata* in grapevine wood

5.1.2.1 Extent of infection in grapevine

In spring 1999, 14 own–rooted Shiraz vines planted in 1967 in the Barossa Valley, South Australia, were flagged as having severe eutypa dieback. All vines exhibited severe stunting of shoots and contained large sections of dead tissue along one or both cordons. In winter 2000, the vines were uprooted and cut into sequential transverse sections to trace colonisation of the below–ground parts of the vine. Discolouration of the wood was recorded and colonisation confirmed by isolation of *E. lata* from symptomatic wood 5 cm above and below soil level.

5.1.2.2 Effect of cultivar on growth rate of E. lata

A field trial was established to determine the growth rate of *E. lata* in grapevine wood. The trial consisted of grapevines (cvs. Cabernet Sauvignon, Gamay, Grenache, Merlot, Pinot Noir, Riesling, Semillon and Shiraz) which were planted between 1974 and 1991 and located at the Nuriootpa Research Centre in South Australia. Fresh wounds on 1–year–old wood were inoculated with a suspension of *E. lata* ascospores in water (1,000 ascospores per wound, five wounds per vine) either on 7 September 2000 or 21 August 2001. Vines were assessed for foliar

symptoms early in spring each year following inoculation. Spurs were removed on 21 July 2004 and returned to the laboratory for assessment. Bark was removed using a knife to reveal the extent of stained wood (Figure 5.1) and then spurs were dissected using loppers and secateurs. To assess the extent of colonisation by *E. lata*, the distance of stained tissue from the inoculation point was measured with a ruler and, to confirm the presence of *E. lata*, tissue from each sample was analysed using the *E. lata*–specific DNA probe (see section 5.4.2). Only samples confirmed to have *E. lata* were considered for statistical analysis using Statistix for Windows (Anonymous 2000).



Figure 5.1. Grapevine spur inoculated with *E. lata* and with bark removed showing the inoculation site (is) and the extent of stained wood (e).

5.1.3 Foliar symptoms

5.1.3.1 Relationship between foliar symptoms and yield

In order to determine the effect of eutypa dieback on grapevine yield, the relationship between foliar symptoms and yield was examined. On 15 December 1999, approximately 125 own–rooted Shiraz vines in each of two vineyards in the Coonawarra region were selected so that about 25 vines fell into each of five disease severity categories. Both vineyards were established in 1968 on a standard T–trellis system and were usually machine–pruned and harvested. Vineyard 1 was drip–irrigated whereas Vineyard 2 had overhead sprinklers.

Disease severity was rated on a 0–4 scale of shoot stunting, where 0 = symptomless and 4 = severe stunting of shoots (Figure 5.2A). Grapes were harvested by hand and the total yield per vine recorded. Vineyard 1 was harvested on 7 March 2000 and Vineyard 2 on 14 March 2000. On 31 October 2000, the same vines were re–assessed for disease severity. Yield data were collected at harvest from Vineyard 1 on 21 March 2001 and from Vineyard 2 on 20 March 2001. Data were subjected to regression analysis using Statistix for Windows. A linear relationship was derived and the equation used to estimate the yield lost to eutypa dieback in each vineyard over the following two seasons.

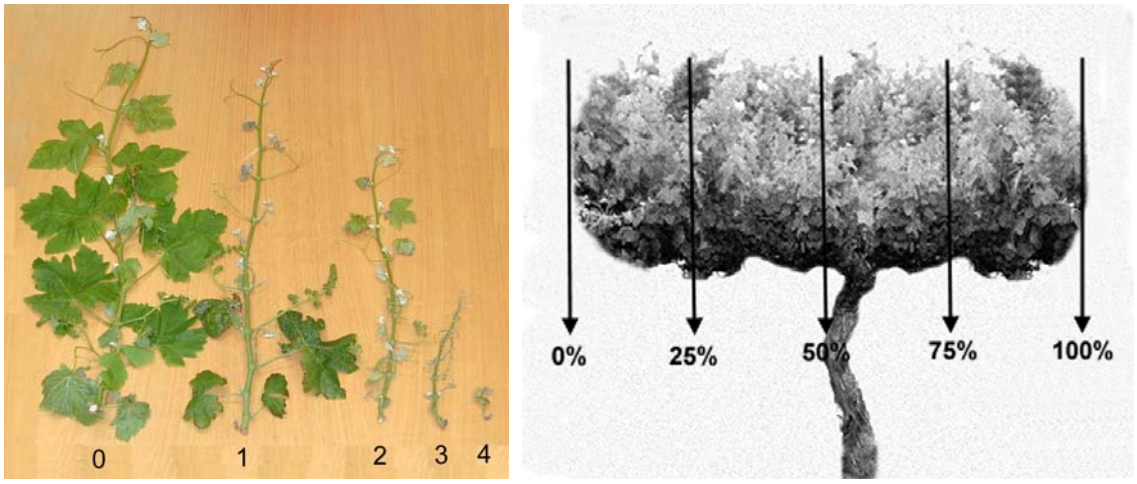


Figure 5.2. eutypa dieback foliar symptom scale developed by T. Wicks (SARDI) based on (A) a severity rating from 0–4 and (B) the percentage of canopy affected.

5.1.3.2 Seasonal variation of foliar symptoms

Five vineyards (cv. Shiraz) were assessed annually in the Coonawarra and Eden Valley regions for foliar symptoms of eutypa dieback for between three and six consecutive years from 1999 to 2004, with the aim of establishing the cause of seasonal variation. A block of between 114 and 144 vines was selected in each vineyard. Symptoms were assessed each spring when shoots were 50–100 cm long, using the foliar symptom severity scale (Figure 5.2A). The percentage of canopy affected was also estimated (Figure 5.2B). Climatic data were collected from automatic weather stations located within 5 km of each trial site. Coonawarra and Eden Valley are approximately 350 km apart and their mean annual rainfalls are 650 and 500 mm, respectively. Four vineyards have standard drip irrigation systems and one Coonawarra vineyard (C1) has overhead sprinkler irrigation.

Three measures of disease intensity were used: (i) disease incidence (DI), which is the percentage of all vines in the trial block that have a symptom rating ≥ 1; (ii) disease prevalence (DP), which is the mean percentage of canopy expressing symptoms in all vines; and (iii) severe disease incidence (SDI), which is the percentage of symptomatic vines that have a rating ≥ 3. Attempts were made to elucidate coincidence between weather parameters (e.g. minimum or maximum temperatures, accumulated temperatures [degree days], rain quantity or number of rain events) and eutypa dieback intensity measures. Analysis was conducted by Prof Dani Shtienberg, Agricultural Research Organisation, Volcani Centre, Israel, during his sabbatical at the University of Adelaide. Only statistically significant relationships are presented in the results.

5.1.4 Pathogenicity of *E. lata* and cultivar susceptibility

A trial was set up to investigate the pathogenicity of Australian isolates of *E. lata* and the susceptibility of grapevine cultivars to both foliar symptoms and colonisation of the fungus in wood. Rooted cuttings (cvs Grenache, Cabernet Sauvignon and Merlot obtained from a commercial nursery in Barmera) were planted in plastic bags filled with potting soil in September 2003. Forty isolates of *E. lata* and eight isolates of other fungi commonly isolated from grapevine trunks were collected from wine regions in South Australia and Victoria and cultured on agar plates. In February 2004 agar plugs (5 mm diameter) containing mycelium were inserted into 5 mm diameter holes drilled in the main stem of the rootlings and sealed by

wrapping with Parafilm (Figure 5.3A). Two separate experiments were established using 768 grapevine plants. In Experiment 1, Grenache vines were treated with 28 isolates of *E. lata*. In Experiment 2, all three cultivars were treated with 12 isolates of *E. lata* and eight non–*E. lata* isolates. Uninoculated control vines, either treated with a sterile agar plug or left untreated, were included in both experiments. There were eight replicates per treatment, including the controls. Following inoculation, vines were watered daily for 7 days to ensure that the inoculation site remained moist. In March 2004 and 2005, vines were pruned to two buds. Foliar symptoms were assessed in October 2004 and 2005, when shoots were 50–70 cm long. Assessments were made using an adapted version of the eutypa dieback foliar symptom severity scale (Figure 5.2), where shoot length for each scale was converted to a percentage of the length of a symptomless shoot.

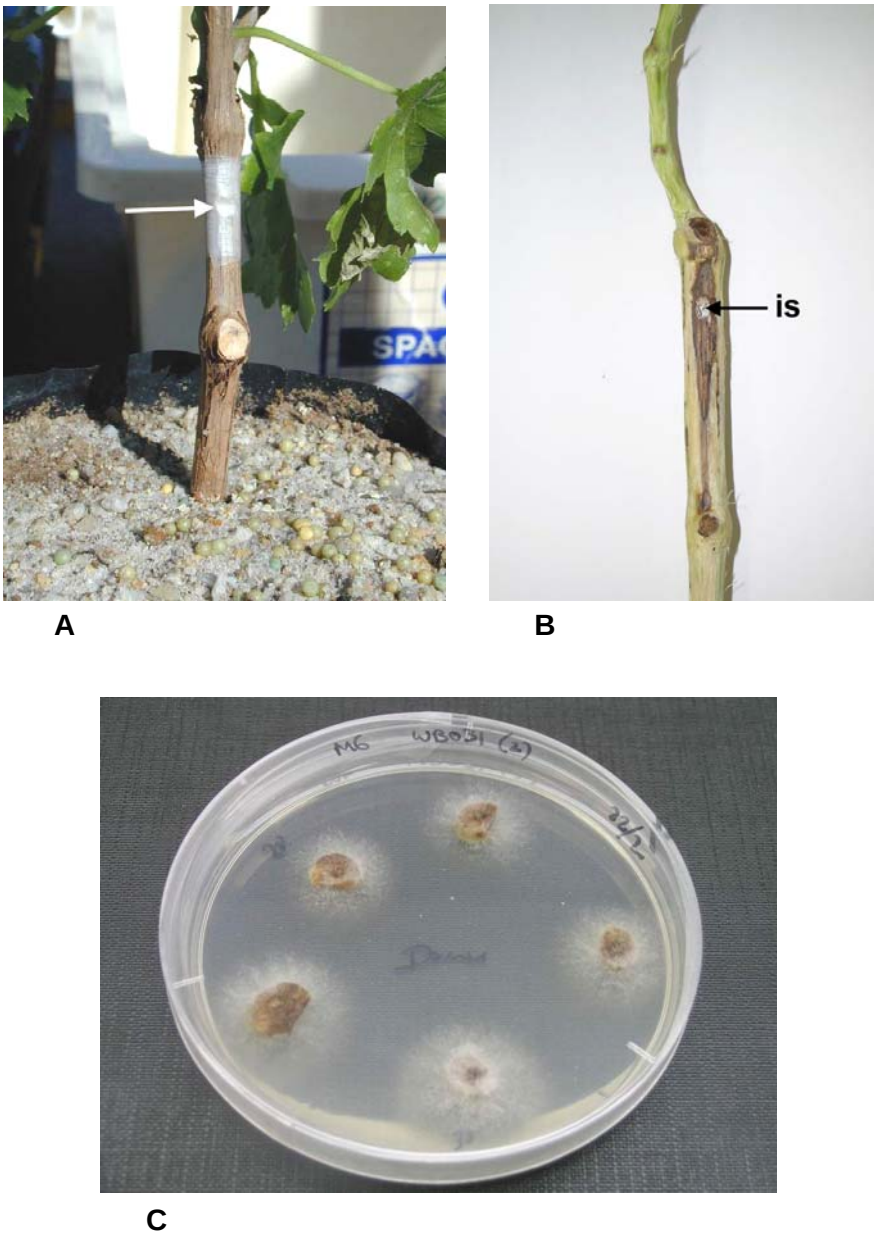


Figure 5.3. (A) Grapevine plant with 5 mm diameter hole filled with agar plug (arrow) and covered with Parafilm; (B) Stem of rooted grapevine cutting following removal of bark, showing the extent of staining above and below the inoculation site (is) and (C) Agar plate with 2 mm sections of a stem sample showing mycelium of *E. lata* growing from each section following 4 days of incubation.

In February 2006 cuttings were taken from the soil, washed and roots were removed. Bark was removed using a knife to expose the extent of stained wood. The extent of colonisation by *E. lata* was assessed by measuring the distance of stained tissue above and below the inoculation point (Figure 5.3B). Each stem was surface–sterilised in a bleach solution for 12 min and, using sterilised secateurs, 2 mm sections were taken at 5 or 10 mm intervals and placed onto plates of potato dextrose agar (PDA) with streptomycin added. (Figure 5.3C). Samples were incubated at 23°C under 100% fluorescent light for 1 week before they were assessed for presence or absence of *E. lata.* Four replicates of each experiment were assessed in this manner.

5.1.5 Survey of Hunter Valley and Mudgee, New South Wales

Grapevines in the Hunter Valley and Mudgee regions of NSW were observed with dieback of cordons and a review of samples received for diagnosis showed no record of isolation of *E. lata* (T. Somers, personal communication 2002). In a survey of Semillon vines in the Hunter Valley, *Botryosphaeria obtusa* was commonly isolated from vines with dead spurs and cordons (Castillo–Pando *et al.*, 2001). However, because symptoms of eutypa dieback are rarely observed in Semillon, a more extensive investigation of the disease was necessary, particularly amongst more susceptible varieties such as Shiraz and Cabernet Sauvignon, in the region.

In collaboration with Dr Sandra Savocchia, Charles Sturt University, vineyards of different ages (from 2 to 100 years old) and varieties (cvs Cabernet Sauvignon, Shiraz, Chardonnay, Semillon and Muscat) in the Hunter Valley and Mudgee wine regions were surveyed for eutypa dieback in early November 2002. Vines in 20 vineyards were inspected for foliar and wood symptoms of the disease. Wood samples were taken from five vines in each vineyard and assessed in the laboratory for the presence or absence of *E. lata* and other fungi by isolation from wood sections on PDA.

5.2 Prevention

5.2.1 Evaluation of treatments for the prevention of wound infection

5.2.1.1 Laboratory evaluation

The effect of 22 treatments (Table 5.1) on ascospore germination and mycelial growth of *E. lata* was evaluated in a series of six *in vitro* experiments in 2000 and 2001.

Table 5.1. Treatments and active ingredients used for *in vitro* experiments in 2000 and 2001 to evaluate efficacy against *E. lata*.

Treatment	Active ingredient
Amistar	Azoxystrobin (500 g/kg)
Cabrio	Pyraclostrobin (250 g/L)
BASF516	Boscalid (200 g/L) + Pyraclostrobin (100 g/L)
Bayfidan	Triadimenol (250 g/L)
Benlate	Benomyl (500 g/kg)
Bravo	Chlorothalonil (500 g/L)
Citowett	Alkylarlyploy glycoether (100 g/L)
Dithane DF	Mancozeb (750 g/kg)
Flint	Trifloxystrobin (500 g/kg)
Fungaflor	Imazalil (750 g/kg)
Kocide	Copper hydroxide (360 g/L)
Nustar	Flusilazole (200 g/kg)
Octave	Prochloraz (462 g/kg)
Ridomil Gold MZ	Mancozeb (640 g/kg)
Rovral	Iprodione (500 g/kg)
Scala	Pyrimethanil (400 g/kg)
Shirlan	Fluazinam (500 g/L)
Teldor	Fenhexamid (500 g/L)
Topas	Penconazole (100 g/L)
Ziram	Ziram (760 g/kg)
Thiovit	Sulphur (800 g/kg)

For each experiment, the effect of fungicide on percentage germination of ascospores was evaluated as follows. Ascospores were liberated from pieces of infected dead wood (5 cm long) with stromata using methods described by Carter (1991). The wood pieces were soaked for 1 h in distilled water and then attached to polypropylene lids (70 mm diameter), which were then screwed onto polycarbonate containers (78 mm depth) and left overnight to allow ascospores to be discharged. The following day the spores were collected by adding sterile distilled water (SDW) to the containers. Ascospores suspended in SDW were spread over PDA plates amended with the prescribed concentration of fungicide and incubated for 2–4 days at 22–25ºC under fluorescent light for 12 h each day. On each plate, 400 spores were viewed under a microscope and the percentage of germinating spores was calculated.

For each experiment the effect of fungicide on mycelial growth was evaluated as follows. Mycelial plugs of *E. lata* were transferred onto PDA from storage and incubated at 22–25ºC under fluorescent light for 12 h each day for 1–2 weeks. Using a cork borer, 5 mm plugs of agar with mycelia were taken from the margin of *E. lata* cultures and placed in the centre of Petri dishes (85 mm diameter) containing PDA amended with the prescribed concentration of fungicide. The plates were then incubated at 22–25ºC under fluorescent light for 12 h each day for between 3 and 9 days. The diameter of the cultures was measured with a ruler and 5 mm was

subtracted to account for the original plug. Five and nine replications were used for Experiments 1 and 2, respectively, with ten replications used in each of Experiments 3–5 and five replications used for Experiment 6. Data were recorded and analysed by ANOVA using Statistix for Windows (Anonymous 2000).

5.2.1.2 Field evaluation

Three trials were established in the 2000–01 growing season to evaluate the efficacy of pruning wound treatments for the control of *E. lata*. Trials 1 and 3 were located at the Nuriootpa Research Centre in South Australia and comprised 95 and 103 Cabernet Sauvignon vines, respectively, planted in 1984. Trial 2 was located in Eden Valley, South Australia, using 114 Shiraz vines planted in 1971.

In 2000, 1–year–old canes were pruned to two buds using secateurs on 1, 3 and 16 August for Trials 1, 2 and 3, respectively. Treatments were applied liberally to ten pruning wounds on each vine with a paint brush within one hour of wounding. Controls, inoculated (I) and not inoculated (NI), were treated with SDW. The following day, five pruning wounds per vine were moistened by spraying with SDW and then inoculated (except NI controls) with 500 *E. lata* ascospores in 10 µl SDW using a pipette. Fourteen days after treatments were applied, the remaining five pruning wounds were inoculated as above. Each trial was set up as a completely randomised design.

Table 5.2. Treatments, active ingredients and rates used for pruning wound protection trials in 2000–01.

Treatment	Active ingredient	Rate
Benlate	Benomyl (500 g/kg)	25 g/L
Solucuivre (France)	Copper and Carbendazim (4%)	40 mL/L
Fungaflor	Imazalil (750 g/kg)	1.36 g/L
Garrison	Paste + cyproconazole (2.5 g/L) + iodocarb (1 g/L)	n/a[a]
Nectec Pasta	Paste + Imazalil (2%)	n/a
Nustar	Flusilazole (200 g/kg)	2.7 g/ L
Scala	Pyrimethanil (400 g/kg)	15 g/L
Shirlan	Fluazinam (500 g/L)	2 mL/L
Topas	Penconazole (100 g/L)	0.25 mL/L
Actizyme	*Bacillus* spp. (including *B. subtilis*)	20 g/L
HayRite	*Bacillus* spp. ($1.3x10^9$ cells/g) (no *B. subtilis*)	20 g/L
Trichoseal Spray	*Trichoderma* spp.	10 g/L
Citowett	Alkylarlyploglycoether (100 g/L)	1 drop/L
ATCS acrylic paint	n/a	n/a
Paint + Benlate	Benomyl	25 g/L
I control[b]	Sterile distilled water	n/a
NI control[c]	Sterile distilled water - no inoculum	n/a

[a] n/a - not applicable.
[b] I control - control treated with water and inoculated with *E. lata.*
[c] NI control - control treated with water and not inoculated.

In July 2001, treated spurs were harvested from vines in all three trials and returned to the laboratory for analysis. Bark was removed from each spur using a sharp knife. The exposed wood was surface–sterilised by flaming and then, using sterilised secateurs, chips ($3 \times 2 \times 2$ mm) were cut from each side of the margin between live and dead wood tissue. Five wood chips were plated onto each plate of PDA, with two plates per spur. Cultures were incubated at 24°C for 7 days and then assessed for presence or absence of *E. lata*. Results were calculated as mean percentage recovery (MPR) of infected spurs. Figure 5.4 illustrates these methods.



A



B



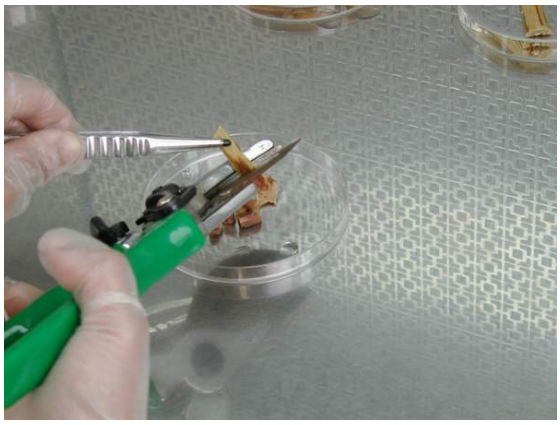
C



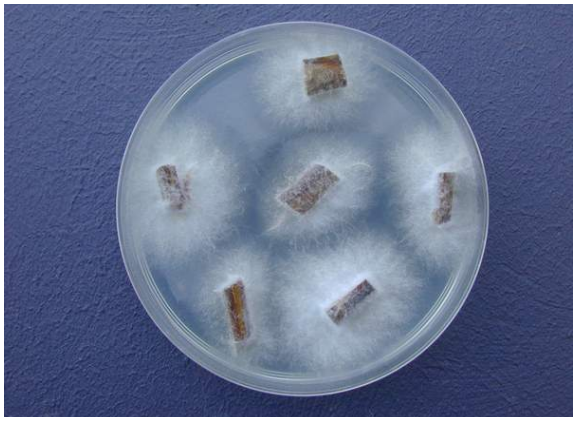
D



E



F



G

Figure 5.4 Evaluation of treatments for the prevention of wound infection: (A) Cutting canes to two-bud spurs on Cabernet Sauvignon vines at Nuriootpa; (B) Applying treatment to wound with a paint brush; (C) Inoculating treated wounds with ascospores of *E. lata*; (D) Spurs removed from vines during the following winter; (E) Spurs with bark removed; (F) Cutting chips from either side of the necrotic margin and (G) Cultures of *E. lata* growing from wood chips.

Two trials (Trials 4 and 5) were established in the 2001–02 growing season to evaluate the efficacy of selected treatments (Table 5.3) for the control of *E. lata*. Trials were located at the Nuriootpa Research Centre in South Australia using Cabernet Sauvignon vines planted in 1984. One–year–old canes were pruned to two buds on 14 August 2001.

Table 5.3. Treatments, active ingredients and rates used for pruning wound protection trials in 2001–02.

Treatment	Active ingredient	Rate
Bavistin	Carbenzidim (500 g/kg)	25 g/L
Fungaflor	Imazalil (750 g/kg)	1.36 g/L
Nustar	Flusilazole (200 g/kg)	2.7 g/L
Scala	Pyrimethanil (400 g/kg)	15 g/L
Shirlan	Fluazinam (500 g/L)	2 mL/L
Topas	Penconazole (100 g/L)	0.25 mL/L
Solucuivre (France)	Copper and Carbendazim (4%)	40 mL/L
ATCS paint + Bavistin	Carbenzidim (500 g/kg)	25 g/L
I control[a]	Sterile distilled water	n/a[b]
NI control[c]	Sterile distilled water - no inoculum	n/a

[a] I control - control treated with water and inoculated with *E. lata.*
[b] n/a - not applicable.
[c] NI control - control treated with water and not inoculated.

Trial 4. Treatments were applied liberally to five pruning wounds per vine using a paint brush within one hour of pruning. A further five wounds per vine were inoculated as above. The following day, treated vines were inoculated (preventative) and inoculated vines were treated (curative) as above. The trial was set up as a randomised block design with five replications using 25 vines.

Trial 5. Treatments were mixed in SDW at three rates of active ingredients (a.i.; 12.5, 6.25 and 3.13 g/L) and applied liberally to 10 pruning wounds per vine using a paint brush within one hour of pruning. The following day, five pruning wounds per vine were inoculated as above and this was repeated 14 days after treatments were applied. The trial was set up as a completely randomised design with five replications using 71 vines. In July 2002, treated spurs were harvested from vines in both trials and returned to the laboratory for analysis as described above for trials in 2002.

Trial 6. In 2002–03, a trial was conducted on Cabernet Sauvignon vines at the Nuriootpa Research Centre by initially pruning 1–year–old canes to two buds on 13 August 2002. Inoculation took place one day before, one day after and 14 days after treatments (listed in Table 5.4) were applied during August 2002. The trial was set up as a randomised block design with 10 replications using 100 vines. Treated spurs were removed in June 2003 and each spur cut into two pieces. One set of samples was evaluated using the methods described above and the other was processed for DNA analysis using protocols described in section 5.4.2. Results from each analysis were compared.

Trial 7. In 2004–05, a trial was conducted on Cabernet Sauvignon vines at the Nuriootpa Research Centre by initially pruning 1–year–old canes to two buds on 13 July 2004. Inoculation, with 1000 ascospores/wound, took place 1 day after treatments (listed in Table 5.5) were applied. However, fruiting bodies on dead wood used to produce inoculum were not correctly identified as *E. lata* and consequently cultures derived from these spores were later identified as belonging to a *Libertella* sp., which has very similar morphology to *E. lata*. Hence a second inoculation was conducted using 1000 ascospores/wound to the same wounds, correctly identified as *E. lata*, 6 days after treatments were applied. The trial was set up as a randomised block design with seven replications using 63 vines. Treated spurs were removed in June 2005 and evaluated as described above (Trial 6).

Table 5.4. Treatments, active ingredients and rates used for pruning wound protection trials in 2002–03.

Treatment	Active ingredient	Rate
BASF 516	Boscalid (200 g/L) + pyraclostrobin (100 g/L)	1.6 mL/L
Bayfidan	Triadimenol (250 g/L)	0.2 mL/L
Benlate	Benomyl (500 g/kg)	2 g/L
Cabrio	Pyraclostrobin (250 g/L)	1.6 mL/L
Flint	Trifloxystrobin (500 g/kg)	0.3 g/L
Teldor	Fenhexamide (500 g/L)	2 mL/L
Thiovit	Sulphur (800 g/kg)	4 g/L
Topas	Penconazole (100 g/L)	0.5 mL/L
I control[a]	Sterile distilled water	n/a[b]
NI control[c]	Sterile distilled water - no inoculum	n/a

[a] I control - control treated with water and inoculated with *E. lata.*
[b] n/a - not applicable.
[c] NI control - control treated with water and not inoculated.

Table 5.5. Treatments, active ingredients and rates used for pruning wound protection trials in 2004–05.

Treatment	Active ingredient	Rate
Bavistin	Carbenzidim (500 g/kg)	2 mL/L
Cabrio	Pyraclostrobin (250 g/L)	1.6 mL/L
Boron	Boric acid	5%
ATCS paint + Bavistin	Carbenzidim (500 g/kg)	20 mL/L
ATCS paint + Boron	Boric acid	5%
ATCS paint	n/a	n/a[a]
Garrison	Paste + cyproconazole (2.5 g/L) + iodocarb (1 g/L)	n/a
I control [b]	Sterile distilled water	n/a
NI control[c]	Sterile distilled water - no inoculum	n/a

[a] n/a - not applicable.
[b] I control - control treated with water and inoculated with *E. lata.*
[c] NI control - control treated with water and not inoculated.

5.2.2 The effect of wound size on efficacy of wound treatments

Trial 1. A trial was established at the Nuriootpa Research Centre to evaluate the effect of wound size on the efficacy of treatments (Table 5.6) for the control of *E. lata*. On 14 August 2001, Gamay vines, planted in 1988, were pruned to provide five pruning wounds of 0.5–1 cm diameter and five wounds of 2–3 cm diameter on each vine. Treatments were applied liberally to ten pruning wounds per vine using a paint brush within one hour of pruning. Controls, I and NI, were treated with SDW. The following day ten pruning wounds per vine were moistened by spraying with SDW and then inoculated (except NI controls) with 500 *E. lata* ascospores in 10 µl SDW using a pipette. The trial was set up as a randomised complete block design with seven replications using 63 vines.

Trial 2. A trial was established in the 2004–05 growing season to evaluate the efficacy of treatments for the control of *E. lata* on large cordon wounds made during vine restructuring. The trial was located at the Nuriootpa Research Centre using 84 Gamay vines planted in 1988.

On 14 August 2004, cordons were cut using electric secateurs or a pruning saw (if greater than 4 cm diameter), ensuring no wedge of discoloured tissue was visible in the cross–section. Treatments (listed in Table 5.7) were applied liberally to the two cordons on each vine using a paint brush within one hour of wounding. Controls, I and NI, were treated with SDW. Seven

days later all pruning wounds were inoculated (except NI controls) by spraying with 25,000 *E. lata* ascospores per mL SDW until run–off occurred, using a spray bottle. The trial was set up as a randomised complete block design using 127 vines and analysed using a one-way analysis of variance using Statistix (Anonymous 2000).

In July 2005, treated cordons were removed from vines using electric secateurs or a pruning saw and samples were returned to the laboratory for analysis. Bark was removed from each sample using a sharp knife. A drill bit (3 mm) was surface–sterilized in ethanol followed by flaming, and drilled into the necrotic tissue near the margin of live tissue. Shavings of necrotic tissue (80 mg) were placed into Eppendorf tubes and then frozen. DNA was extracted from samples and slot blot analysis was used to detect the presence of *E. lata* as described in section 5.4.2.

Table 5.6. Treatments, active ingredients and rates used for Trial 1 to assess the protection of different sized wounds in 2001–02.

Treatment	Active ingredient	Rate
Bavistin	Carbenzidim (500 g/kg)	25 g/L
Fungaflor	Imazalil (750 g/kg)	1.36 g/L
Trichoseal spray	*Trichoderma* spp.	10 g/L
Scala	Pyrimethanil (400 g/kg)	15 g/L
Garrison	Paste + cyproconazole (2.5 g/L) + iodocarb (1 g/L)	n/a
ATCS paint	n/a[a]	n/a
ATCS paint + Bavistin	Carbenzidim (500 g/kg)	25 g/L
I control[a]	Sterile distilled water	n/a
NI control[c]	Sterile distilled water - no inoculum	n/a

[a] I control - control treated with water and inoculated with *E. lata.*
[b] n/a - not applicable.
[c] NI control - control treated with water and not inoculated.

Table 5.7. List of treatments, active ingredients and rates used for cordon wound protection in Trial 2 in 2004–05.

Treatment	Active ingredient	Rate
Bavistin	Carbenzidim (500 g/kg)	2 mL/L
Cabrio	Pyraclostrobin (250 g/L)	1.6 mL/L
ATCS paint	n/a[a]	n/a
Garrison	Paste + cyproconazole (2.5 g/L) + iodocarb (1 g/L)	n/a
Vinevax	*Trichoderma* spp. (500 million cfu[b]/g)	100 g/L
Kocide	Cupric hydroxide (500g/kg)	8 g/L
Scorch	n/a (wound burnt with flame)	n/a
control[c]I	Sterile distilled water	n/a
NI control[d]	Sterile distilled water - no inoculum	n/a

[a] n/a - not applicable.
[b] cfu - colony forming units.
[c] I control - control treated with water and inoculated with *E. lata.*
[d] NI control - control treated with water and not inoculated.

5.2.3 The efficacy of spray application of wound treatments

Trial 1. A trial was established in the 2001–02 growing season to evaluate the efficacy of selected treatments (Table 5.3) infected by *E. lata* when applied with a hand–held sprayer in comparison with application with a paint brush. Trials were located at the Nuriootpa Research Centre in South Australia using Cabernet Sauvignon vines planted in 1984. One–year–old canes were pruned to two buds on 14 August 2001. In July 2002, treated spurs were harvested from vines in each trial and returned to the laboratory for analysis as described above for trials in 2002.

Treatments were applied liberally to 15 pruning wounds per vine, either using a paint brush or with a hand–held sprayer within one hour of pruning. The following day, five pruning wounds per vine were inoculated as above and this was repeated 7 and 14 days after treatments were applied. The trial was set up as a split–plot design with three replications using 72 vines.

Trial 2. In 2002–03, a trial was conducted on Cabernet Sauvignon vines at the Nuriootpa Research Centre to assess the efficacy of spray application by initially pruning 1–year–old canes to two buds using secateurs. Benlate (2 g/L) was applied to pruned vines with a sprayer attached to the back of a four–wheel motorbike. Other treatments included painting Benlate onto wounds, I and NI controls. Treatments were applied on 27 August and wounds were inoculated with ascospores 1 and 14 days later, as described above. Treated spurs were removed in June 2003 and evaluated as described above.

Trial 3. In 2004–05, a trial was set up on Cabernet Sauvignon vines at the Nuriootpa Research Centre to assess the efficacy of spray application by initially pruning 1–year–old canes to two buds on 5 August 2004. Bavistin (5 mL/L) was applied to pruned spurs using either a Hardi air–blast sprayer (310 L/ha), a SARDI fan sprayer (596 L/ha) or by hand with a paint brush. I and NI controls were also included. Treated wounds (except NI controls) were each inoculated with 500 ascospores in 20 µL of water 1 day after treatment. However, fruiting bodies on dead wood were not correctly identified as *E. lata* and consequently spores were identified as belonging to a *Libertella* sp. Hence a second inoculation was conducted using 1000 ascospores/wound, correctly identified as *E. lata*, 14 days after treatments were applied. Treated spurs were removed in June 2005 and evaluated as described above.

5.2.4 Residue analysis

5.2.4.1 Benlate (a.i. benomyl)

Two trials were conducted at the Nuriootpa Research Centre to determine if Benlate applied as a wound protectant to prevent infection by *E. lata* in winter resulted in residues in fruit at harvest. In the plant, the main breakdown product of benomyl is carbendazim.

In August 1999, half of the vines in a commercial vineyard in Nuriootpa, South Australia were restructured. Following surgery, large wounds on each of the 27–year–old Shiraz vines were treated immediately with 0.7 g/L Benlate from a hand–held pressure sprayer. The remaining vines were restructured in July 2000 and treated in the same way. In March 2001, five vines were selected at random from each half of the vineyard and fruit collected, pooled according to treatment date, and tested for carbendazim residues at the Australian Wine Research Institute (AWRI).

In August 2000, two wounds, measuring 2–3 cm in diameter, were made on each of 15 12–year old spur–pruned Gamay vines, and one of the following treatments was applied to both wounds on each vine using a brush within 30 min of wounding, in a completely randomised design. The treatments were; control treated with water, 25 g Benlate per L water (12.5 g/L benomyl) and 50 g Benlate per L water (25 g/L benomyl). Fruit was harvested from each vine in February 2001 and tested for carbendazim residues as above.

5.2.4.2 *Garrison (a.i. cyproconazole + iodocarb)*

A trial was established in the 2003–04 growing season to evaluate the efficacy of Garrison for the control of *E. lata* on large cordon wounds made during vine restructuring. The trial was located at the Nuriootpa Research Centre using 84 Gamay vines planted in 1988. On 25 February 2004, grape bunches were collected from vines treated with Garrison 7 months previously for residue testing. Garrison was applied liberally with a paint brush until the wound was completely covered. Three samples (2 kg of grapes each) were collected from across the trial, combining several replications for each sample (GA 1-3). Another 2 kg sample was collected from six replications of untreated controls (GA 4). All four samples were analysed for cyproconazole residues at the AWRI, however, no analysis was available for iodocarb.

5.3 Control

5.3.1 Remedial surgery

There is no reliable means to eradicate *E. lata* once it becomes established within a vine. Consequently, many growers "renew" infected vines by removing infected wood and reworking or top–working. When infection is restricted to one or both cordons, wood is removed until the characteristic cross–sectional wedge of discoloured wood is no longer visible and then a further 10–20 cm of apparently healthy wood is removed to ensure that mycelium is eradicated. A cane is then trained to replace the section of cordon.

Where cordons are infected and the fungus has progressed into the trunk, two methods are used to restore vines:

1. The cut and train (C&T) method involves cutting off the trunk 30–40 cm above the ground, then inducing and training up a watershoot to replace the lost canopy (Figure 5.5A). Stumps can also be grafted.

2. The train and cut (T&C) method, where a healthy shoot is selected from the base of the trunk and trained up to form a new canopy (Figure 5.5B). The infected trunk is not removed until the new shoot begins cropping.



A B

Figure 5.5. (A) the cut and train method and (B) the train and cut method of reworking a vine.

Twelve remedial surgery trials were established on several grapevine cultivars in a number of growing regions over five years of the project. In some trials, foliar symptoms and/or wood symptoms in trunk cross–sections were recorded during surgery. In Trial 1, wounds were treated with either Benlate, Nustar or Trichoseal. In Trials 2 and 3, wounds were treated with Benlate mixed into paint and in Trials 4 and 5 wounds were treated with Benlate in water. In Trials 6–8, wounds were treated with Benlate mixed into acrylic paint or with Garrison. In Trial 9, wounds were treated with Benlate, Garrison, Vinevax, acrylic paint or left untreated as controls followed by inoculation with ascospores of *E. lata*. In Trial 10, wounds were treated with Garrison. In Trial 11, 205 vines were injected with StemShot GN1 and 150 control vines were not injected. For StemShot treated vines, wounds were covered with Vinevax paste and for control vines wounds were sprayed with Spin-flo. In Trial 12, vines were cut back until stained wood was no

longer visible, and Vinevax was applied to fresh wounds.

The objective of these trials was to assess the effectiveness of remedial surgery, which is determined by restoration of the vine to productivity (yield and quality), recurrence of symptoms and increased longevity of the vines. For each trial, the percentage of watershoot production and foliar symptom recurrence was recorded during spring in the following seasons.

For Trials 7 and 8, wounds were also assessed for the presence of wood discolouration typical of eutypa dieback, esca and Petri disease. Samples (2–3 cm slices of trunk) were collected for fungal isolation and molecular diagnosis of *E. lata*.

5.3.2 Grafting

A grafting trial was set up at the Nuriootpa Research Centre in the Barossa Valley to assess Sauvignon Blanc vines which were cut 30–40 cm above the ground and cleft–grafted with Shiraz (two canes per vine) on 19 October 2001. The wound was cut, prised open with a chisel, then tissue paper and two Shiraz canes were inserted into the cleft. Wounds were painted with wound treatments; Bavistin (12 mL/L), Fungaflor (2.8 g/L), Nustar (8.1 g/L), Scala (30 g/L) or Trichoseal (g/L) as well as I and NI controls, and sealed with a waterproofing emulsion, Bitkote (Davco Construction Materials Pty Ltd). On the following day, wounds were inoculated with 5,000 ascospores of *E. lata*. Each spring following grafting, vines were assessed for graft success and appearance of foliar symptoms of eutypa dieback.

5.3.3 Injection trials

Trials were established in 2001 to evaluate four experimental chemicals applied by trunk injection for the management of eutypa dieback–affected grapevines. The system was developed by Chemcolour Industries (NZ) Ltd. The four chemicals (commercial in confidence) and water (control) were injected into own–rooted Shiraz vines on two commercial properties in the Eden Valley (planted in 1971) and the Coonawarra (planted in 1970). The incidence and severity of foliar symptoms of eutypa dieback was assessed before injection and each spring for the following three seasons using the rating system described in Figure 5.2. Yield, number of bunches and average bunch weight were assessed for each vine at harvest.

5.3.4 Brotomax trials

The nitrogen–based foliar fertilizer Brotomax was evaluated for control of eutypa dieback. Brotomax was applied to own–rooted Shiraz (planted in 1971) and Cabernet Sauvignon (1960) vines on two commercial properties in Eden Valley and McLaren Vale, respectively, over four growing seasons between 2001 and 2005. Vines naturally infected with *E. lata* were sprayed at (i) shoot length 20–30 cm, (ii) 20–25 days later, (iii) bunch closure and (iv) 15–20 days after harvest. Brotomax (10 mL/L) was applied until run–off (approx. 1.5 L) to the foliage and trunk of each vine using a backpack sprayer (Figure 5.6). Yield, number of bunches and average bunch weight per vine were assessed at harvest. Disease severity was assessed in late spring using the rating scale described in Figure 5.2. Since the number of vines for each level of severity was not equal, the data were subjected to Restricted Maximum Likelihood analysis using GenStat–Release 7.1.



Figure 5.6. Spraying Brotomax on vines using a backpack spraying unit

5.3.5 *Trichoderma* trials

Five field trials were established to assess the ability of *Trichoderma* spp. to control eutypa dieback. Trial 1 was set up in the winter of 1996 on Shiraz vines planted in 1971 at Eden Valley. Trial 2 was set up in 1996 on Cabernet Sauvignon vines planted in 1962 at Reynella. Both vineyards displayed foliar symptoms of eutypa dieback. Treatments included injection of Trichoseal (*Trichoderma* spp.) and benomyl along with insertion of Trichodowels (wooden dowels impregnated with *Trichoderma* spp., Figure 5.7) and blank wooden dowels as a control. Trial 3 was also established in Eden Valley during 1996 on Shiraz vines (planted in 1918) and severely affected by eutypa dieback. Trunks were treated by injection with Trichoseal and controls remained untreated. Trial 4 was located in the Clare Valley on Cabernet Sauvignon vines planted in 1981. In 2002, Trichodowels were inserted into trunks of vines with foliar symptoms of eutypa dieback and control vines remained untreated. In 2003, Trial 5 was established on vines planted in 1992 with no foliar symptoms of eutypa dieback. Trunks were either treated with Vinevax biodowels or left untreated as controls.

Each trial was assessed for foliar symptoms of eutypa dieback using the rating scale described in Figure 5.2. Trial 1 was assessed in the spring of 1996, 1997 and 1998, Trial 2 was assessed in 1996 and 1997 and Trial 3 was assessed in 1996.



Figure 5.7. Insertion of a dowel impregnated with *Trichoderma* spp. into the trunk of a vine.

5.4 Diagnosis of eutypa dieback

5.4.1 Development of DNA markers specific to *E. lata*

E. lata has traditionally been identified by isolating the pathogen in pure culture and identifying based on colony morphology. However, this is time–consuming and other grapevine–inhabiting fungi may be indistinguishable from *E. lata* in culture. A major aim of the research was to develop molecular techniques for the rapid and reliable identification and detection of *E. lata*. Two approaches were taken to generate molecular markers specific to *E. lata*, as described below. Once validated, the markers were used to detect *E. lata* direct in infected wood and to identify the pathogen from mycelium. Fungal isolates used in this study are given in Appendix 6. A comprehensive description of this research is given in the PhD thesis of Lardner (2003) (available online at thesis.library.adelaide.edu.au/public/adt-SUA20050608.143820/index.html).

5.4.1.1 Development of SCAR markers for a PCR–based assay

DNA was isolated from mycelium of selected isolates of *E. lata* and amplified using the RAPD (random amplified polymorphic DNA) technique as described by Lardner *et al.* (2005). Two DNA fragments apparently specific to *E. lata* were cloned and sequenced, and two sets of SCAR (sequence characterised amplified region) primers, one specific to each DNA fragment, were designed and validated using DNA from three isolates of *E. lata*. One primer pair (designated Eut02 F3/Eut02 R2) was selected for further validation using DNA from an additional 21 isolates of *E. lata* and 12 other fungi isolated from grapevine, as described by Lardner *et al.* (2005).

5.4.1.2 Assessment of SCAR and ITS markers developed in France and California

Six PCR primer pairs developed in France (Lecomte *et al.*, 2000) were tested on DNA from 24 isolates of *E. lata* and 14 other grapevine inhabiting fungi from Australia. Reactions were set up using the conditions described by Lecomte *et al.* (2000), with each reaction performed at least twice.

A pair of *E. lata*–specific PCR primers developed by Californian researchers at E. & J. Gallo Winery were obtained under a Material Transfer Agreement. The primer pair was tested on DNA from the same isolates used to test French primer pairs, using the proprietary protocols of E. & J. Gallo Winery.

5.4.1.3 *Development of markers for a Southern hybridisation–based assay*

An *E. lata* genomic DNA library was constructed by digesting genomic DNA from *E. lata* isolate M280 with *Pst*I restriction enzyme and ligating into the plasmid vector pBluescript, using methods described in Sambrook *et al.* (1989). Southern hybridisation was used to evaluate five clones for their specificity towards the pathogen, using DNA from 23 isolates of *E. lata*, 14 non–*E. lata* isolates and grapevine DNA, as described by Lardner *et al.* (2005). One clone, designated C68, was selected for use in further experiments.

5.4.2 Detection of *E. lata* in grapevine wood using DNA markers

SCAR primer pair Eut02 F3/Eut02 R2 and RFLP probe C68 were used to detect *E. lata* in 1–year–old cane segments, cuttings or mature grapevine trunks as outlined below. Unless otherwise stated, mycelium of *E. lata* was recovered from each sample as described by Carter (1991).

5.4.2.1 *PCR–based detection of* E. lata

Eight protocols were used to extract DNA from infected grapevine wood for use in PCR, namely

the Bio-101 FastDNA SPIN kit for soil (Qbiogene), the DNeasy Plant Mini Kit (Qiagen), the modified DNeasy protocol of Green *et al.* (1999), the rapid extraction protocol of Lecomte *et al.* (2000), the CTAB–based protocol of Wolf *et al.* (1999), the proprietary method developed at E. & J. Gallo Winery, the SEAPS protocol of Melanson *et al.* (2002) and the silica–based protocol of Rott and Jelkmann (2001). Amplification of DNA by PCR was carried out as described by Lardner *et al.* (2005).

Automated high–throughput DNA extraction procedures used at the Australian Genomic Research Facility (AGRF), based at the Waite Campus, were also evaluated for their ability to extract high quality DNA of *E. lata* from infected wood for use in fluorescence–based PCR analysis. This was carried out by researchers at the AGRF using their proprietary techniques.

5.4.2.2 Southern hybridisation–based detection of E. lata

DNA clone C68 was used in a slot blot format to detect *E. lata* in infected grapevine wood as described by Lardner *et al.* (2005). The slot blot assay was initially validated using DNA isolated from 10 grapevine canes which had been inoculated with *E. lata*, and two uninoculated control canes, as well as using DNA isolated from 11 symptomatic, naturally infected grapevine trunks.

The probe was then comprehensively validated for use with mature wood using samples taken from the trunks of 476 vines being re–worked in vineyards in the Eden Valley, McLaren Vale and Coonawarra regions (see section 5.3). For each sample, the presence of *E. lata* was determined by culturing mycelium from wood chips and by slot blot analysis.

Finally, the probe was validated for use on annual cane using DNA isolated from 1250 samples from pruning wound trials on annual canes. Pruning wounds had been inoculated with ascospores of *E. lata* and treated with water or one of eight different fungicides (section 5.2.1.2). For each sample, the presence of *E. lata* was determined by culturing mycelium and by slot blot analysis.

5.4.3 Identification of *E. lata* from mycelium using SCAR markers

SCAR markers were used to identify *E. lata*, without the need for DNA extraction, by boiling mycelium in extraction buffer and adding an aliquot of the resulting suspension direct to the amplification reaction, as described by Hamelin *et al.* (2000). The protocol was validated using mycelium from 26 authenticated isolates of *E. lata* and 14 non–*E. lata* isolates. Subsequently, mycelium from 80 putative *E. lata* isolates obtained from the trunks of 198 43–year–old Shiraz grapevines in McLaren Vale was tested.

5.4.4 Genetic variation in *E. lata*

Genetic variation in *E. lata* was assessed using RAPD and RFLP analysis. Comparisons were made of data from RAPD, RFLP and secondary metabolite analysis to evaluate correlations among these factors.

5.4.4.1 RAPD analysis

Eleven isolates received as *E. lata*, which were used in a preliminary analysis of secondary metabolite production, were screened with 10 RAPD primers. RAPD–PCR amplification was conducted as described by Péros *et al.* (1996). Data were imported into the Treecon package (Van de Peer and De Wachter, 1994) and analysed using the neighbour joining technique.

5.4.4.2 RFLP analysis

Thirty eight isolates from Australia and other countries were subjected to RFLP analysis using

six randomly selected clones from the *E. lata* genomic DNA library, as described by Lardner (2003). Data were analysed using the neighbour joining technique.

Each of the 38 isolates subjected to RFLP analysis was included in the analysis of secondary metabolites described below. To evaluate relationships between genetic variation within *E. lata* and secondary metabolite production, secondary metabolite data were also analysed using neighbour joining, and results of molecular and biochemical analyses were compared.

5.4.5 Production of phenolic secondary metabolites by *E. lata*

Based on reports that eutypine caused the foliar symptoms of eutypa dieback and could be detected in the sap and inflorescences of infected vines (Tey–Rulh *et al.*, 1991), an original aim of project 2.2.1 was to develop a rapid diagnostic test for eutypa dieback based on detecting eutypine in infected vines. However, researchers at the United States Department of Agriculture (USDA) showed that not all isolates of *E. lata* produced eutypine in artificial culture (Molyneux *et al.*, 2002). Based on these reports, and because secondary metabolite production by Australian isolates of the pathogen had not been assessed, we conducted a preliminary analysis of secondary metabolite production using 11 isolates received as *E. lata*, including six isolates from Australia. The aim of this was to identify culture media conducive to the production of secondary metabolites and to clarify the types of metabolite produced by *E. lata*. This research was conducted in collaboration with Dr Russell Molyneux at the United States Department of Agriculture in California. As part of this research, Richard Lardner was awarded a CRCV visiting scholars award to spend 3½ months working in the laboratory of Dr Molyneux, to become conversant with techniques required for analysis of secondary metabolites by *E. lata*.

Based on information gathered in the preliminary experiments, we then analysed secondary metabolite production by a further 30 isolates of *E. lata* grown on three cultivars of grapevine wood, using HPLC to identify an appropriate chemical marker for the disease. HPLC was also used to detect such metabolites *in planta*. Separate experiments were conducted to see if eutypine could be detected in infected vines or in pure cultures of the pathogen using Raman spectroscopy or thin layer chromatography (TLC). Raman Spectroscopy and TLC analysis was conducted at Monash University. Methods used in these experiments are outlined below. Identification of culture media and preliminary assessment of secondary metabolite production by *E. lata*

Secondary metabolite production on artificial media

Eleven isolates (E1, E120, E125, E178, M266, M279, M280, M335, N04, SS1#1 and SS6) received as *E. lata* from France, Italy, America, New Zealand and Australia were grown on four artificial liquid media, namely potato dextrose broth (PDB), malt yeast broth (MYB), Vogel's Medium (Vogel, 1964) and Pezet's medium (Pezet, 1983), maintained in shaken and stationary conditions. Secondary metabolite production was also assessed on two solid media (PDA and Vogel's agar). All cultures were kept in the dark at 25ºC. Secondary metabolites were extracted and analysed using HPLC as described by Molyneux *et al.* (2002). Two of the isolates (E178 and SS1#1) were later found not to be *E. lata*, based on biochemical and molecular analysis.

Secondary metabolite production on grapevine extracts

Because of variation in secondary metabolite production on artificial media, secondary metabolite production by the same 11 isolates was assessed following incubation on media derived from grapevine canes. This was expected to approximate the nutrient conditions to which the fungus is naturally exposed and, hence, provide a more representative indication of secondary metabolite production by *E. lata* in infected vines. Grapevine–based media consisted of ground grapevine cane, ground grapevine cane supplemented with sucrose, liquid grapevine cane extract, liquid grapevine cane extract supplemented with sucrose, liquid grapevine cane

extract solidified with agar and liquid grapevine cane extract supplemented with sucrose and solidified with agar. Details of medium preparation are given in Lardner (2003) and Mahoney *et al.* (2003). Secondary metabolites were extracted and analysed using HPLC as described by Molyneux *et al.* (2002).

5.4.5.1 Identification and characterisation of a specific chemical marker

Identification of metabolites specific and ubiquitous to E. lata

Preliminary experiments showed that secondary metabolite production was more consistent on grapevine extracts than on the artificial media tested. To identify chemical markers appropriate for use in an early diagnostic test, a further 30 isolates of *E. lata* were grown on wood from three cultivars with varying tolerance to eutypa dieback (Shiraz, susceptible; Merlot and Semillon, tolerant). It was hoped that this would test whether cultivar tolerance to eutypa dieback influences secondary metabolite production. Metabolites were extracted from 20–day–old cultures grown on ground grapevine cane amended with sucrose and analysed using HPLC. The identity of all isolates was confirmed using the SCAR markers specific to *E. lata*.

To confirm that metabolites were specific to *E. lata*, a further 18 isolates, comprising five isolates of *Phaeomoniella chlamydospora*, three isolates each of *Botryosphaeria* sp. and *Fomitiporia sp*., two isolates each of *Cryptovalsa ampelina* and *Phaeoacremonium aleophilum* and one isolate each of *Fomitiporia punctatus*, *Botryosphaeria ribis* and *Libertella* sp., were also incubated on ground wood (cv. Shiraz) for 20 days and culture filtrates were analysed using HPLC.

Phytotoxicity of secondary metabolites and correlation with pathogenicity in the shadehouse

To help identify the metabolites responsible for the foliar symptoms of eutypa dieback, the phytotoxicity of total secondary metabolite extracts from the 11 isolates grown in the four artificial liquid media and on ground grapevine cane (section 5.4.5) was analysed by applying purified filtrates to grapevine leaf discs as described by Lardner (2003) and Smith *et al.* (2003).

To see if there was a correlation between pathogenicity and secondary metabolite production *in vitro*, a comparison was made between the secondary metabolite profiles of 12 isolates grown on ground cane and the pathogenicity of each isolate to potted vines (cv. Grenache) in the shadehouse (see section 5.1.4).

Rapid detection of E. lata *with spectroscopy and chemometrics*

The acetylenic phenol metabolites produced by *E. lata* have an unusual structure with series of conjugated double bonds, triple bonds and aldehyde groups (Tabacchi, 1994, Molyneux et al., 2002). This gives them a strong and unique UV spectral fingerprint and offers an opportunity for rapid screening with minimal sample preparation using spectroscopy combined with chemometric methods.

To determine the feasibility of such an approach, two flasks of each of 20 isolates of *E. lata* were grown on MYB at 25°C for 20 days in the dark. Replicate cultures were combined and metabolites were extracted using di–ethyl ether, as described in section 5.4.5. Metabolites were re–suspended in 2 mL methanol, and a 200µl portion of each extract was set aside for HPLC analysis as described previously. The remainder was analysed using UV–Vis spectroscopy as follows: The culture extracts were further diluted in methanol (200 µl extract + 2.8 mL methanol) to get the spectra on scale, then scanned on a Varian *Cary 300* spectrophotometer (1 cm path 200–400 nm, 1 nm interval, 10 scan averaging), using a methanol reference. Spectra were exported as *GRAMS* files into *The Unscrambler* for chemometric analysis.

Spectra were examined “raw”, or modified with Savitsky–Golay smoothing plus second derivative transformation (6 point data gap). Wavelengths <240 nm were off–scale with some samples, so 240–400 nm was used for principal component analysis (PCA).

Detection of eutypine by Raman spectroscopy and thin layer chromatography

Based on reports that eutypine caused the foliar symptoms of eutypa dieback, and because European researchers reported detecting eutypine in the foliage of vines naturally infected with *E. lata* (Tabacchi, 1994), Raman spectroscopy and TLC were used to detect eutypine in the foliage of infected vines.

- Raman spectroscopy

The Raman profile of pure eutypine was assessed using a Renishaw model 2000 Ramanscope at Monash University, which was also used to detect eutypine in leaves, stems and sap taken from vines showing symptoms of eutypa dieback.

- Thin layer chromatography

TLC was used to assess eutypine production by 19 isolates of *E. lata* grown for 14, 42, 56 and 70 days in Pezet’s culture medium. Metabolites were extracted into ethyl acetate, loaded onto silica gel plates and separated in chloroform:ethyl acetate.

Because *E. lata* may produce eutypine more consistently in plant tissue than *in vitro*, leaves of four replicate cuttings, which had been inoculated with one of five isolates of *E. lata* in the glasshouse in South Australia, were extracted and analysed using TLC as described above. In addition, TLC was used to analyse metabolites extracted from two–node cuttings of Chardonnay, Pinot Noir, and Sauvignon Blanc which had been inoculated, individually, with eight isolates of *E. lata* in the glasshouse at Monash University.

Influence of temperature and water availability on secondary metabolite production by E. lata

Analysis of foliar symptom and climatic data (section 5.1.3.2) generated several hypotheses to explain seasonal variation in the incidence and severity of foliar symptoms of eutypa dieback. Rainfall and temperature were implicated as major influences on such variation.

An experiment was set up to assess the effects of temperature and water availability on growth rate and secondary metabolite production by *E. lata in vitro*. Six isolates were selected based on their ability to produce secondary metabolites in culture and their pathogenicity in the shadehouse. Isolates were incubated at 12.5, 17.5, 25 or 30ºC for 20 days on grapevine extract media adjusted with polyethylene glycol to give matric potentials of –0.27, –1, –2, –3 and –6 mPa (S. Tyerman, *pers.com.*). Three replicate plates of each isolate were included for each temperature/matric potential combination. Linear growth rate of cultures was assessed between days four and seven by measuring colony diameter along two axes perpendicular to each other and recording the average of the two measurements as the radial colony diameter. Secondary metabolites were extracted from each culture using di–ethyl ether and analysed using HPLC.

5.4.5.2 *Identification of chemical marker(s) in treated and infected vines*

To gather information on detecting secondary metabolites in grapevine tissue, experiments were conducted using micropropagated vines, vines held in a shadehouse and naturally infected vines in the field. Vines were assessed for the presence of metabolites of *E. lata* using HPLC.

Detection of secondary metabolites in micropropagated vines treated with metabolites of E. lata

In a preliminary experiment, four shoots taken from micropropagated plantlets (cv. Cabernet

Sauvignon) were treated with eutypinol, eulatachromene, eutypine or siccayne, suspended in ethylene glycol. Shoots were exposed to metabolites for 10 days then ground in liquid nitrogen, and metabolites were extracted with di–ethyl ether and analysed using HPLC.

In a second experiment, micropropagated shoots and plantlets (cv. Cabernet Sauvignon) were treated with purified or crude culture filtrates, with known composition of secondary metabolites, from nine isolates of *E. lata*. Filtrates were derived from isolates grown on MYB for 30 days at 25ºC in the dark. Plantlets were exposed to filtrates for 10 days then ground in liquid nitrogen, and metabolites were extracted using di–ethyl ether and analysed using HPLC.

Because the experiment described above showed that metabolites of *E. lata* may be converted to other compounds *in planta*, a time–course experiment was initiated using shoots taken from micropropagated plantlets (cv. Cabernet Sauvignon). Shoots were exposed to purified culture filtrates from ten isolates of *E. lata* for 1, 3, 5, 7 or 10 days (three replicates of each isolate, a total 165 samples including controls) and metabolites were extracted and analysed using HPLC. It was hoped that this would provide information on the longevity and degradation of metabolites *in planta*.

Detection of metabolites in micropropagated vines inoculated with E. lata

Micropropagated grapevine plantlets (cv. Cabernet Sauvignon) were inoculated with mycelium from the nine confirmed isolates of *E. lata* previously analysed for secondary metabolite production (section 5.4.5.1 and Mahoney *et al.*, 2003), by applying mycelium to wounds made in the stems of plantlets, as described by Lardner *et al.* (2006). After 42 days, inoculated plantlets were ground in liquid nitrogen and metabolites were extracted using di–ethyl ether.

Because foliar symptoms were not expressed consistently in the above experiment, various protocols were tested to induce typical symptoms of eutypa dieback on micropropagated material. This included manipulating humidity, growth medium, temperature, photoperiod and inoculation technique, as well as using isolates of *E. lata* shown to be pathogenic in the shadehouse (Sosnowski *et al.*, 2005). We also established a collection of micropropagated Grenache plantlets, derived from plants used in shadehouse pathogenicity experiments, given the increased tendency of this cultivar to display foliar symptoms in the shadehouse. Metabolites were extracted from material used in these experiments using di–ethyl ether and analysed using HPLC.

HPLC analysis of extracts from inoculated and naturally infected vines

Sap was taken from 30 potted vines that had been inoculated 21 months previously with mycelium from characterised isolates of *E. lata* in the shadehouse (section 5.1.4). Ten of these vines, each inoculated with a different isolate, displayed characteristic foliar symptoms of eutypa dieback 8 months after inoculation; a further ten vines, inoculated with the same ten isolates, were asymptomatic. The remaining ten vines were non–inoculated or inoculated negative controls (blank PDA plugs). Metabolites were extracted from the sap using di–ethyl ether and analysed using HPLC.

Metabolites were extracted from foliage taken from naturally infected vines in the field (cv. Cabernet Sauvignon) displaying symptoms of eutypa dieback. Foliage was ground in liquid nitrogen and metabolites isolated using four different protocols. These included the protocol of Afifi *et al.* (2004) and the protocol of Mahoney *et al.* (2003), using either di–ethyl ether, chloroform or ethyl acetate as the solvent.

6 RESULTS AND DISCUSSION

6.1 Epidemiology

6.1.1 Ascospore inoculum

6.1.1.1 Ejection of ascospores

Colonies of *E. lata* resulted from 42 and 13% of the wood chips placed 5 and 10 mm below the agar, respectively. The wood of Shiraz bore fewer stromata than that of Grenache, and the perithecia from Shiraz produced slightly fewer colonies than those from Grenache.

6.1.1.2 Effect of rainfall on ascospore release and germination

The absence of colonies from dry wood confirmed the requirement for moisture to initiate ascospore release. As the amount of simulated rainfall increased so too did the number of wood chips which gave rise to colonies of *E. lata* (Table 6.1). Fewer colonies arose from stromata on Grenache exposed to 1 mL of simulated rainfall than to 2 mL, whereas the number from stromata on Shiraz was similar for 1 and 2 mL of rainfall. Application of 20 mL of simulated rainfall increased the number of colonies arising from stromata on Shiraz compared to 2 mL of rainfall, but not for Grenache.

Table 6.1. The effect of simulated rainfall on ascospore production from two grapevine cultivars.

Cultivar	Vol (mL)	No. samples producing *E. lata* colonies	Total no. samples	% samples producing *E. lata* colonies
Grenache	0	0[a]	40	0
	1	8[b]	40	20
	2	17[c]	44	39
	20	19[c]	40	47
Shiraz	0	0[a]	40	0
	1	10[b]	42	24
	2	10[b]	36	28
	20	17[c]	40	42

Values with the same superscript are not significantly different at P = 0.05

6.1.1.3 Interaction between distance of ascospore ejection and temperature

The number of wood chips bearing stromata that ejected ascospores decreased significantly as the distance to the layer of PDA increased from 2 to 10 mm (Figure 6.1). Increasing the temperature from 4 to 22ºC had no significant effect on the number of wood chips resulting in colonies on the agar.

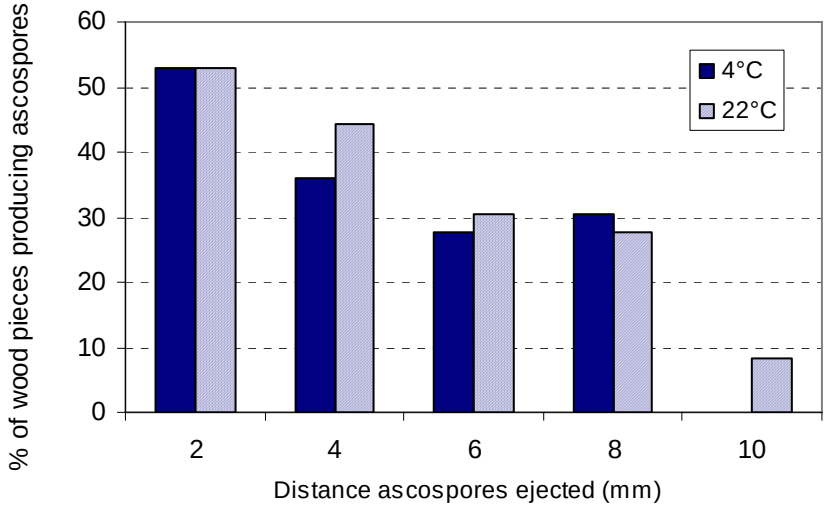


Figure 6.1. Distance of ascospore ejection from stromata on pieces of grapevine wood at two temperatures. $LSD_{0.05}$ = 9.

There were significant differences in ascospore ejection between the two cultivars tested. Release of *E. lata* ascospores was greater for stromata on Grenache than on Shiraz. The wood of Grenache vines also bore more stromata. These observations were consistent for all experiments and support the conclusion that Grenache is highly susceptible (Carter 1991).

Rainfall has been observed to affect the amount and timing of ascospore release (Carter 1957, Carter 1991, Emmett and Hamilton 1994). Here, ascospore release was shown to be highly dependent on rainfall. As the volume of simulated rainfall increased, the number of colonies arising from ejected ascospores also increased, whereas lack of moisture prevented ascospore release. Results showed that 1 mm of rainfall can initiate ascospore release, which is less than previous reports that a minimum of 2 mm is necessary (Carter 1991). There was no difference in spore release at 4 and 22^{o}C, confirming previous reports that temperature has little effect on spore release.

6.1.2 Growth of *E. lata* in grapevine wood

6.1.2.1 Extent of infection in grapevine

Wood symptoms extended below ground in all 14 vines. The average length of the below–ground trunk was 35 cm, which accounted for about 36% of the trunk, when measured from the base of the trunk to the crown of the vine (Figure 6.2). Discoloured wood extended an average of 14 cm into the trunk below ground, to a maximum of 24 cm below soil level. In all vines, discolouration was confined to the trunk tissue and was not observed in the roots.

Of the 14 vines examined, colonisation of the below–ground trunk ranged from 17 to 67%. This variation may relate to the age or original site of infection, or the virulence of the pathogen. As discolouration was not observed in the root system there is no evidence to support transmission of the disease by root to root contact. Such vines, however, are unlikely to be restored to productivity by reworking.

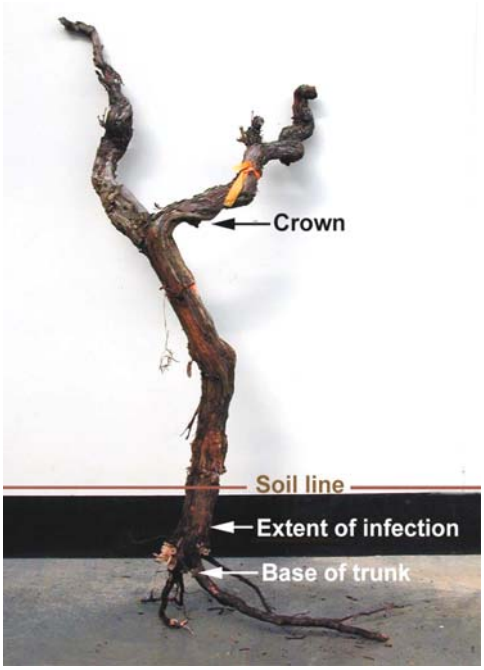


Figure 6.2. Vine showing the average extent of *E. lata*–infected wood in the below ground trunk.

6.1.2.2 Effect of cultivar on growth rate of E. lata

No foliar symptoms of eutypa dieback were observed on any of the mature vines inoculated with *E. lata* for the duration of the trial. Figure 6.3 shows the results of sample assessments and reveals that growth rate of *E. lata* differed significantly among cultivars, ranging from 10 mm/yr (cvs Merlot, Gamay, Grenache and Semillon) to 18 mm/yr (cvs Cabernet Sauvignon and Shiraz). However, it is important to note that there was some variation and there were cases where the fungus progressed at as much as 70, 50 and 40 mm/yr in Shiraz, Pinot Noir and Cabernet Sauvignon vines, respectively.

Staining of wood attributed to necrosis caused by colonisation by *E. lata* progressed at 10–18 mm/yr in mature field vines. Merlot, Gamay, Grenache and Semillon appeared to be more resistant to the spread of staining than Cabernet Sauvignon, Shiraz and Pinot Noir. With the exception of Grenache, these results concur with reports of susceptibility to the foliar symptoms of eutypa dieback (Carter, 1991; Highet and Wicks, 1998). One reason for the discrepancy may be that only eight samples of Grenache were included in the assessment, compared with 18–80 samples for the other cultivars. In this experiment, colonisation by *E. lata* ahead of the staining was not assessed and, as a result, the mean growth rate of *E. lata* maybe greater than reported here.

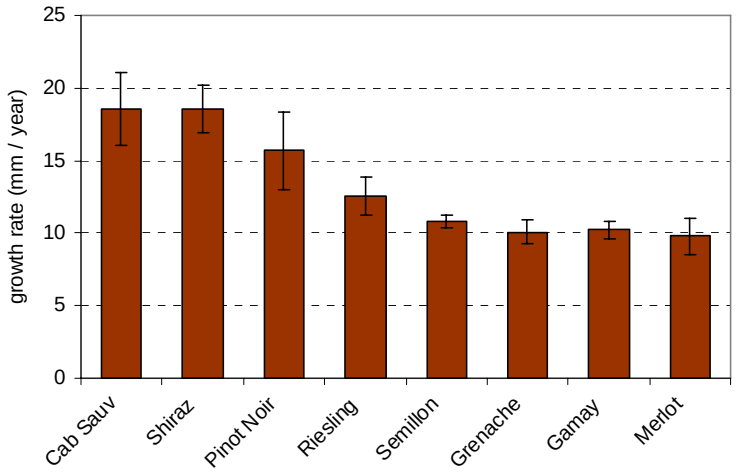


Figure 6.3. Annual growth rate of *E. lata* in grapevine cultivars in the field over 3 or 4 years. Bars represent standard error of the mean

6.1.3 Foliar symptoms

6.1.3.1 Relationship between foliar symptoms and yield

Regression analyses of the data from 1999 and 2000 demonstrated significant linear relationships ($P < 0.05$) between disease severity and yield per vine in both vineyards and in both years (Figure 6.4). The yield per vine decreased as disease severity increased. To facilitate comparisons between vineyards and years of assessment, data from each vineyard were adjusted to a percentage value based on the average yield of a symptomless vine in that vineyard and in that year (Munkvold *et al.*, 1994). When the data were re–analysed, there was no significant difference between vineyards or years of assessment, and the relationship between yield (Y) and disease severity (x) could be described by a single line: Y(% of symptomless) = 101.7 – 17.1x, where x = the mean disease rating for each vineyard in each year. Using this equation, we calculated the yield lost to eutypa dieback in each vineyard over 4 years (Table 6.2). Yield lost to eutypa dieback ranged from 10 to 32% and was significantly greater in 2000 than in subsequent years in both vineyards.

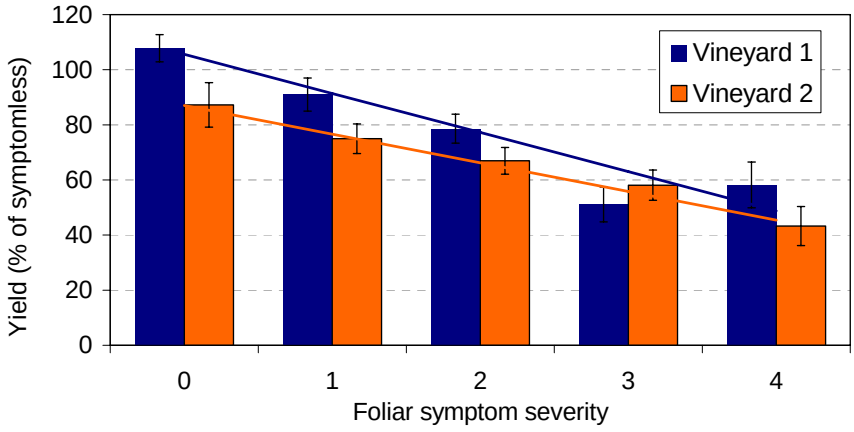


Figure 6.4. Relationship between symptom expression and yield per vine in two vineyards in the Coonawarra when data are expressed as a percentage of the estimates for symptomless vines for 1999–2000 and 2000–01.

Table 6.2. Estimated yield loss in two vineyards in the Coonawarra over 4 years using the equation Y(% of symptomless) = 101.7 – 17.1x, where x = the mean disease rating.

Vineyard	2000	2001	2002	2003
1	32.3%	21.4%	20.2%	17.0%
2	31.9%	14.9%	12.8%	10.0%

Eutypa dieback was shown to be responsible for significant yield reduction in two Shiraz vineyards in the Coonawarra. The relationship between disease severity and yield was consistent between the two vineyards over two years of the study and suggests that it may be possible to predict the yield lost to eutypa dieback by assessing foliar symptoms in the spring prior to harvest. As expected, based on the reduction in severity of foliar symptoms, the yield was significantly greater in 2003 than in the previous 3 years.

6.1.3.2 Seasonal variation of foliar symptoms

In accordance with previous reports, incidence and severity of foliar symptoms varied from year to year. In general, similar trends were observed for all vineyards, however, some differences between regions were apparent. For example, disease incidence decreased by 10 to 30% in all vineyards between 2001 and 2002. However, between 2002 and 2003 there was a 30 to 40% increase in disease incidence in the Eden Valley vineyards, although incidence in the Coonawarra vineyards increased by only 10% (Figure 6.5).

A number of possible relationships between climate and symptom expression were identified. An increase in disease incidence was associated with the number of rain events occurring around the time of mechanical pruning in the previous winter ($year_{i-1}$) (Figure 6.6A). Conversely, a decrease in disease incidence was positively related to spring temperature; the higher the temperature, the fewer vines expressing foliar symptoms (i.e. the more vines "escaped" disease symptoms when they had symptoms in $year_{i-1}$). Similar trends were observed in both regions (Figure 6.6B).

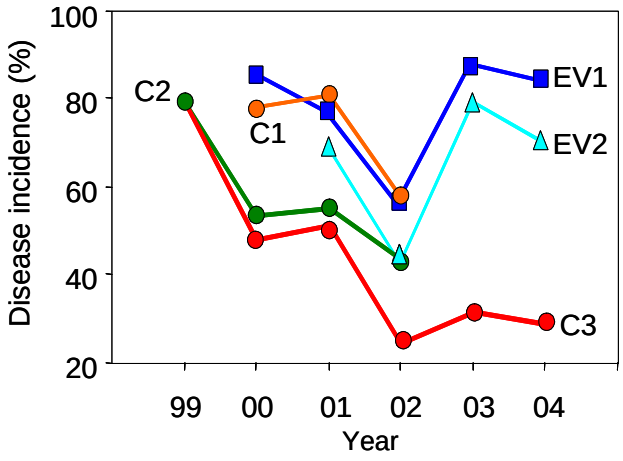


Figure 6.5. Variation of eutypa dieback disease incidence (rating ≥ 1) for all vines in five vineyards located in Eden Valley (EV) and Coonawarra (C) over 6 years.

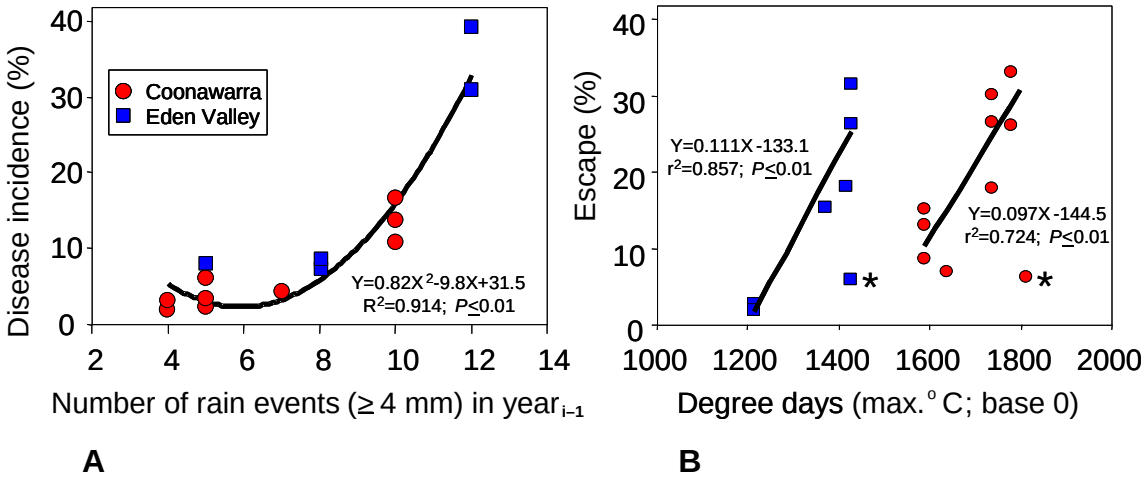


Figure 6.6. Relationship between weather variables and the increase or decrease in the incidence of vines exhibiting symptoms of eutypa dieback. (A) Relationship between the number of rain events (≥ 4 mm) in $year_{i-1}$ and the incidence of new symptoms (i.e. vines that were symptomless in $year_{i-1}$ but exhibited eutypa dieback symptoms in $year_i$). Rainfall events were calculated during the periods 8 June–8 July (Eden Valley) and 20 June–20 July (Coonawarra). (B) Relationship between temperatures in the spring and the incidence of vine recovery (i.e. vines that exhibited disease symptoms in $year_{i-1}$ but were symptomless in $year_i$). Degree days were calculated for August to October (Eden Valley) and September to November (Coonawarra). Data points accompanied by asterisks were considered as outliers and were not included in the regression analyses.

A conceptual model was developed from these data to predict disease incidence. It involves knowledge of disease incidence in the previous year (DI_{i-1}), prediction of new infections (NI_{i-1}) by the rainfall events of the previous winter and the 'escape' from disease symptoms (E_i) by spring temperature.

$$\mathbf{DI_i = DI_{i-1} + NI_{i-1} - E_i}$$

When the model was applied to the climatic data for the two locations, a very similar trend was observed between the predicted and observed incidences. However, it is important to note that this model must be tested on a different set of data before any conclusions can be made about its accuracy.

Disease prevalence and severity were also related to weather variables. For each vineyard, disease prevalence decreased by 0.06% with each degree–day increase in the spring (Figure 6.7A). The relationship between rain in Oct and the incidence of severely infected vines is shown in Figure 6.7B. It reveals that the greater the Oct rainfall, the lower the disease severity. However, it also shows that when Oct rainfall was very low, symptom severity was reduced in the Eden Valley vineyards.

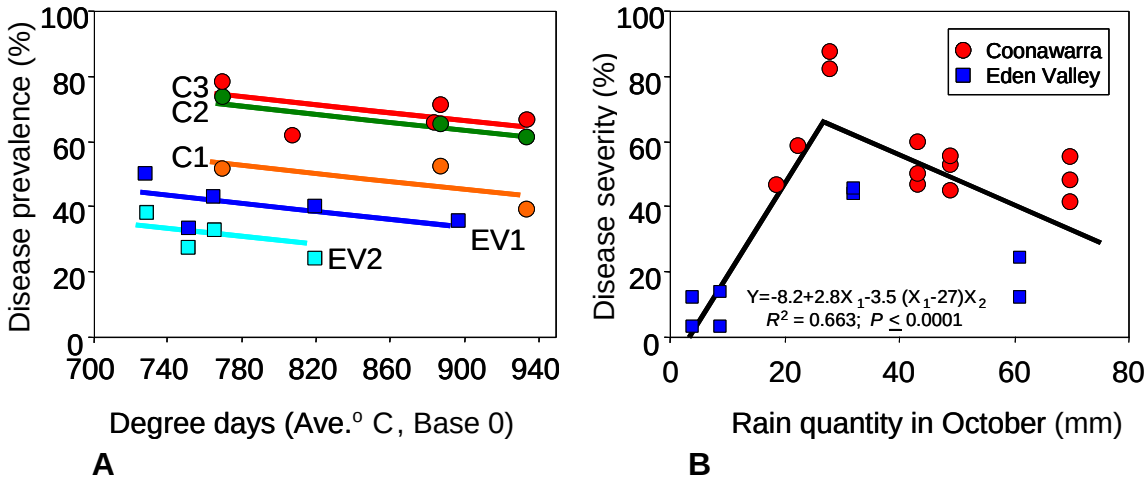


Figure 6.7. Relationship between weather variables and eutypa dieback prevalence and severity. (A) Relationship between spring temperature (October and November) and disease prevalence for each of the five vineyards. Data were analysed using co–variance model (R^2 = 0.923; P < 0.0001). (B) Relationship between rain in October and the incidence of vines exhibiting severe eutypa dieback symptoms (rating > 3) using piecewise regression analysis.

The variation in foliar symptoms of eutypa dieback from year to year may be due to climatic factors rather than vineyard management practices, such as pruning. The different trends observed between the regions may also be due to climatic variation. A number of relationships have been observed, and a number of theories proposed to explain them. However, it is important to note that these relationships may be coincidental and further investigation will be necessary to verify them.

Increased disease incidence between years appeared to be due to increased rainfall in the previous winter. This may be linked to the influence of rainfall on airborne inoculum, but would depend on pruning being conducted within this period. However, symptom expression has not been recorded within 15 months of infection in the field. Decreases in disease incidence and prevalence were related to increased temperature in spring. Vines grow more vigorously in warmer conditions, which could reduce the effect of fungal toxins on the foliage. Alternatively, the production of toxins by *E. lata* may be reduced in warmer conditions. It is also possible that a combination of these reasons may explain the relationship. The most interesting relationship observed in this study involved the effect of October rain on severe disease incidence of eutypa dieback. There may be a complex interaction between host and pathogen which leads to a reduction in disease incidence when moisture availability is high, possibly through toxin dilution, and then a reduction of disease incidence during periods of very low moisture availability, which may be due to reduced toxin production due to water stress on the fungus or limited water movement for transport of toxins through the vine.

6.1.4 Pathogenicity of *E. lata* and cultivar susceptibility

Foliar symptoms typical of eutypa dieback were evident on some treated vines 8 months after inoculation (Figure 6.8A). Foliar symptoms were observed on shoots both above and below the site of inoculation on three cuttings (two Grenache and one Cabernet Sauvignon, Figure 6.8B). Twenty–six of the 40 *E. lata* isolates induced symptoms on at least one of the eight replicate Grenache cuttings. Six isolates, from Eden Valley, Clare, Loxton, Barossa Valley in SA and Strathbogie Ranges in Victoria, induced foliar symptoms on over 50% of the inoculated vines. The mean foliar symptom rating on Grenache varied from 0 to 12% for all isolates. In Experiment 2, foliar symptoms were induced by two *E. lata* isolates in all three cultivars, by four isolates in Cabernet Sauvignon and five isolates in Grenache. Statistical analysis revealed that

the mean foliar rating for Grenache was significantly greater than that for Cabernet Sauvignon or Merlot. Symptoms did not develop in vines inoculated with other wood–inhabiting fungi or in uninoculated controls in either experiment (Figure 6.9).



Figure 6.8. (A) eutypa dieback symptoms on Grenache with characteristic chlorotic, cupped leaves with tattered margins. (B) Grenache vine inoculated with an *E. lata* isolate from Coonawarra, SA, displaying foliar symptoms on shoots extending from both above and below the inoculation site (arrow).

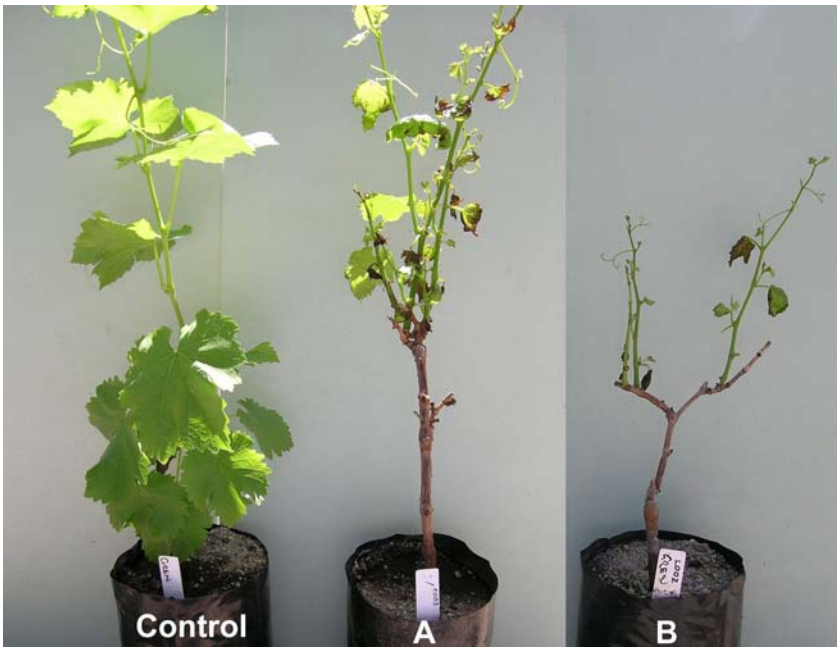


Figure 6.9. Uninoculated control Grenache vine (left) compared to the symptomatic shoots of vines inoculated with *E. lata* isolates from Loxton (A) and Clare (B) in SA.

Vines were also assessed for foliar symptoms during the second spring, 20 months after inoculation. In Experiment 1, 24 of the 28 isolates induced foliar symptoms on Grenache vines,

with severity ranging from 2 to 36% (Figure 6.10). Mycelium of each isolate was recovered from inoculated cuttings following plating of stem sections onto agar. Depending on the isolate, between 4 and 146 mm of grapevine stem (up and down from the inoculation site) was colonised over the 20–month period. Statistical analysis revealed significant differences among isolates for both the severity of symptoms induced and growth rate. However, there was no correlation between the length of wood colonised and the severity of foliar symptoms. There was an effect of region on foliar symptom severity (Figure 6.11), with isolates from Barossa Valley, Loxton and Eden Valley causing more severe foliar symptoms on rootlings than those from Clare, McLaren Vale and Coonawarra. However, no regional effect was evident for colonisation by *E. lata*. Staining spread from 10 to 85 mm/yr for all isolates and growth rate of the fungus ranged from 5 to 67 mm/yr, with nine isolates of *E. lata* advancing ahead of the stain by 5–40 mm over 2 years, with a maximum distance of 67 mm recorded.

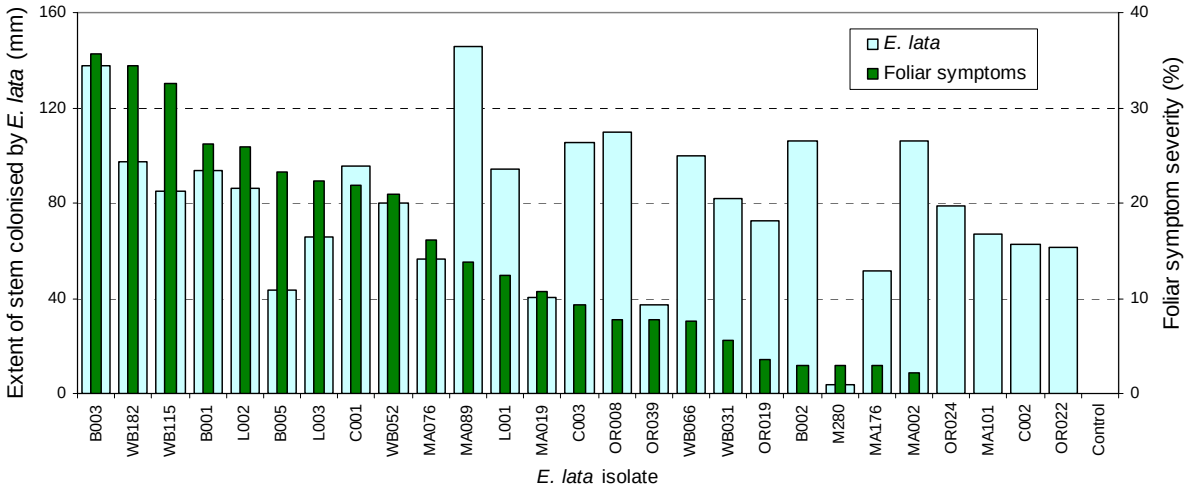


Figure 6.10. Colonisation of grapevine stems by *E. lata* and severity of foliar symptoms on Grenache vines in Experiment 1 in the shadehouse, 20 months after inoculation with *E. lata*. Controls were inoculated with a blank agar plug.

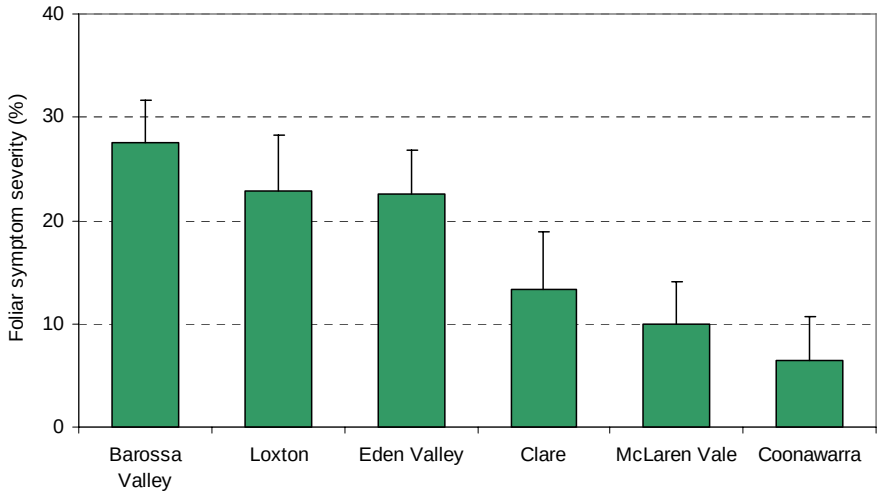


Figure 6.11. Mean severity of foliar symptoms, induced by isolates of *E. lata* from six regions in South Australia, on Grenache vines in Experiment 1 in the shadehouse, 20 months after inoculation.

In Experiment 2, seven of the 12 isolates induced foliar symptoms on Grenache and two isolates induced symptoms on Cabernet Sauvignon and Merlot (Figure 6.12). Only one isolate induced

foliar symptoms on all three cultivars. The mean severity of foliar symptoms was significantly greater for Grenache than for Cabernet Sauvignon or Merlot, however, no significant difference was observed among isolates. Staining was observed on wood inoculated with isolates of each of the fungal species assessed as well as the control which was inoculated with a blank agar plug (Figure 6.13). The extent of staining and spread of mycelium of the 12 isolates of *E. lata* both above and below the inoculation site on each cultivar is illustrated in Figure 6.14. There was significantly more staining in Grenache than in the other two cultivars, however no differences were observed among isolates in any cultivar. Conversely, there was no significant difference among cultivars for the extent of colonisation of tissue by mycelium of *E. lata*, although there were significant differences in the extent of colonisation among isolates. For Cabernet Sauvignon and Merlot, most isolates colonised canes in advance of the staining, however, only five isolates did so in Grenache. After 2 years, the greatest mean distance colonised by mycelium of *E. lata* in advance of stained tissue was 46 mm in Cabernet Sauvignon, 35 mm in Merlot and 34 mm in Grenache. It is important to note that the single sample from which *E. lata* was isolated furthest ahead of staining was 80 mm in Grenache. The average rate of spread of wood staining downwards from the inoculation site ranged from 0–16, 0–45 and 8–52 mm/yr in Cabernet Sauvignon, Merlot and Grenache, respectively and the average growth rate of *E. lata* in the wood was 0–34, 0–29 and 0–41 mm/yr, respectively.

Of the four replicates assessed for colonisation by *E. lata*, foliar symptoms were observed on shoots originating from below the inoculation site (Figure 6.8b) in seven cases. In four of these cases, *E. lata* was isolated 125–195 mm below the inoculation site and in the other three *E. lata* was isolated from 30–40 mm below the inoculation site.

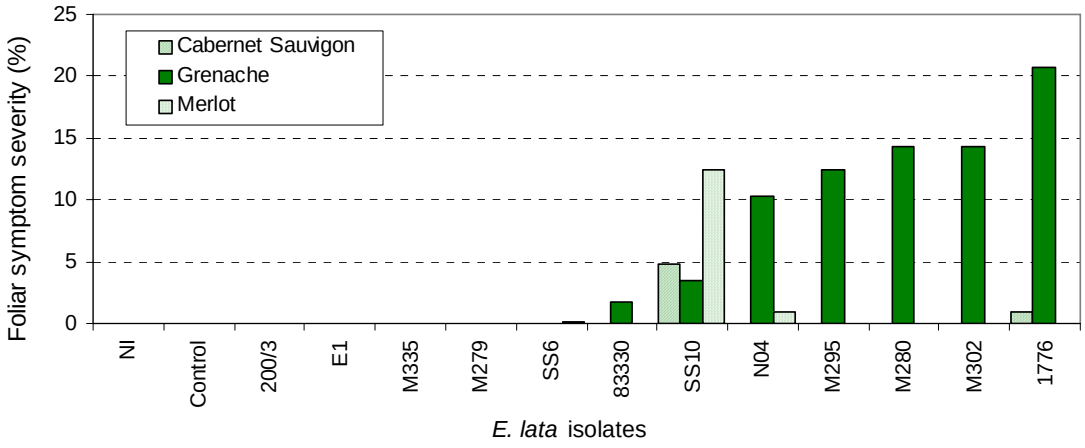


Figure 6.12. Foliar symptom severity (%) on grapevines in Experiment 2 in the shadehouse, 20 months after inoculation with a range of *E. lata* isolates. NI = non–inoculated, Controls were inoculated with a blank agar plug.

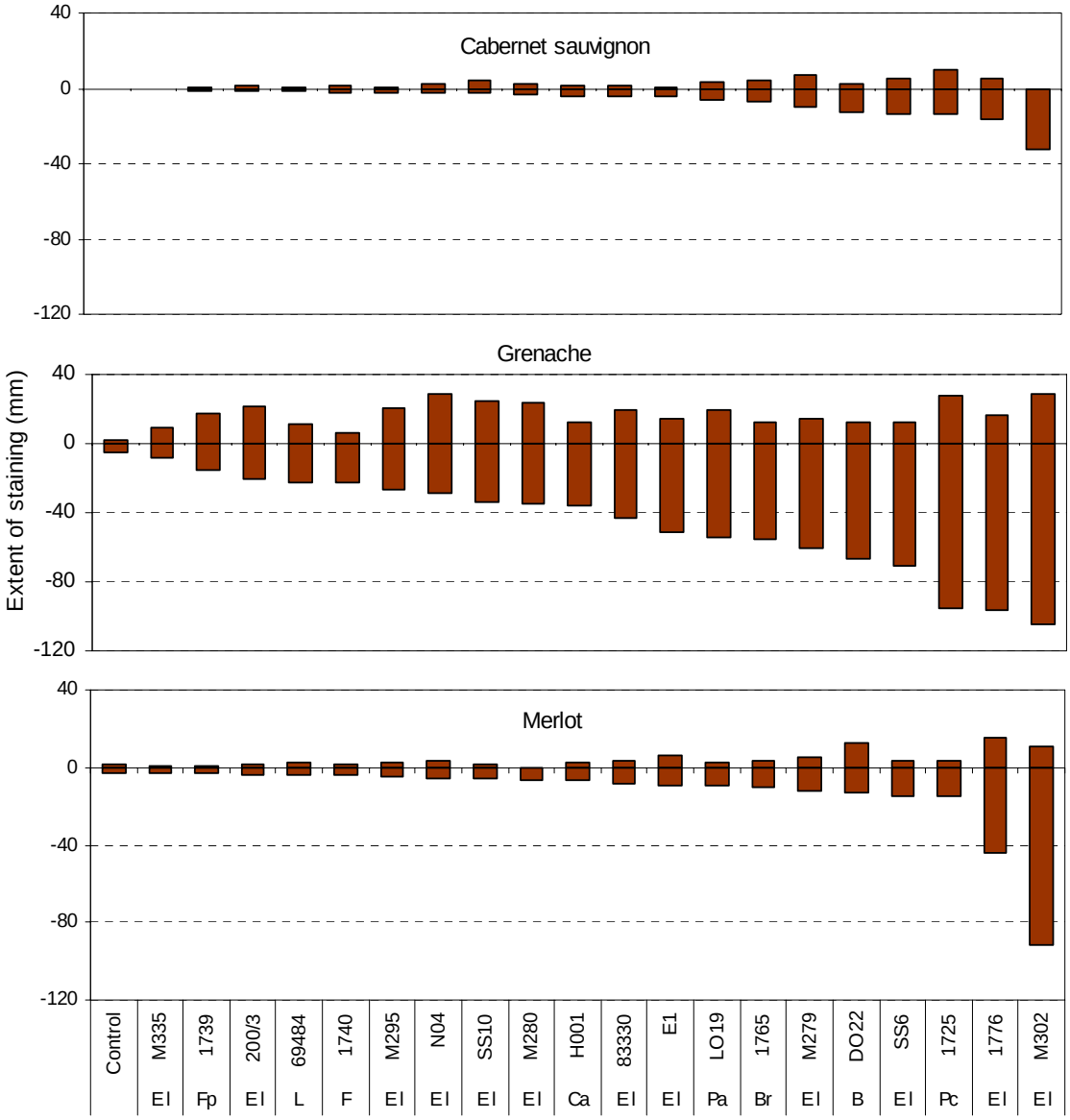


Figure 6.13. Extent of staining by isolates of *E. lata* (*E. lata*), *Fomitiporia punctatus* (Fp), *Fomitiporia* sp. (F) *Libertella* sp. (Lib. sp.), *Cryptovalsa ampelina* (Ca), *Phaeoacremonium aleophilum* (Pa), *Botryosphaeria ribis* (Br), *Botryosphaeria* sp. (B) and *Phaeomoniella chlamydospora* (Pc) above and below (-) the inoculation site (0) on stems of three grapevine cultivars in Experiment 2 in the shadehouse, 20 months after inoculation with each of the isolates. Controls were inoculated with a blank agar plug. Values are the average of four replications.

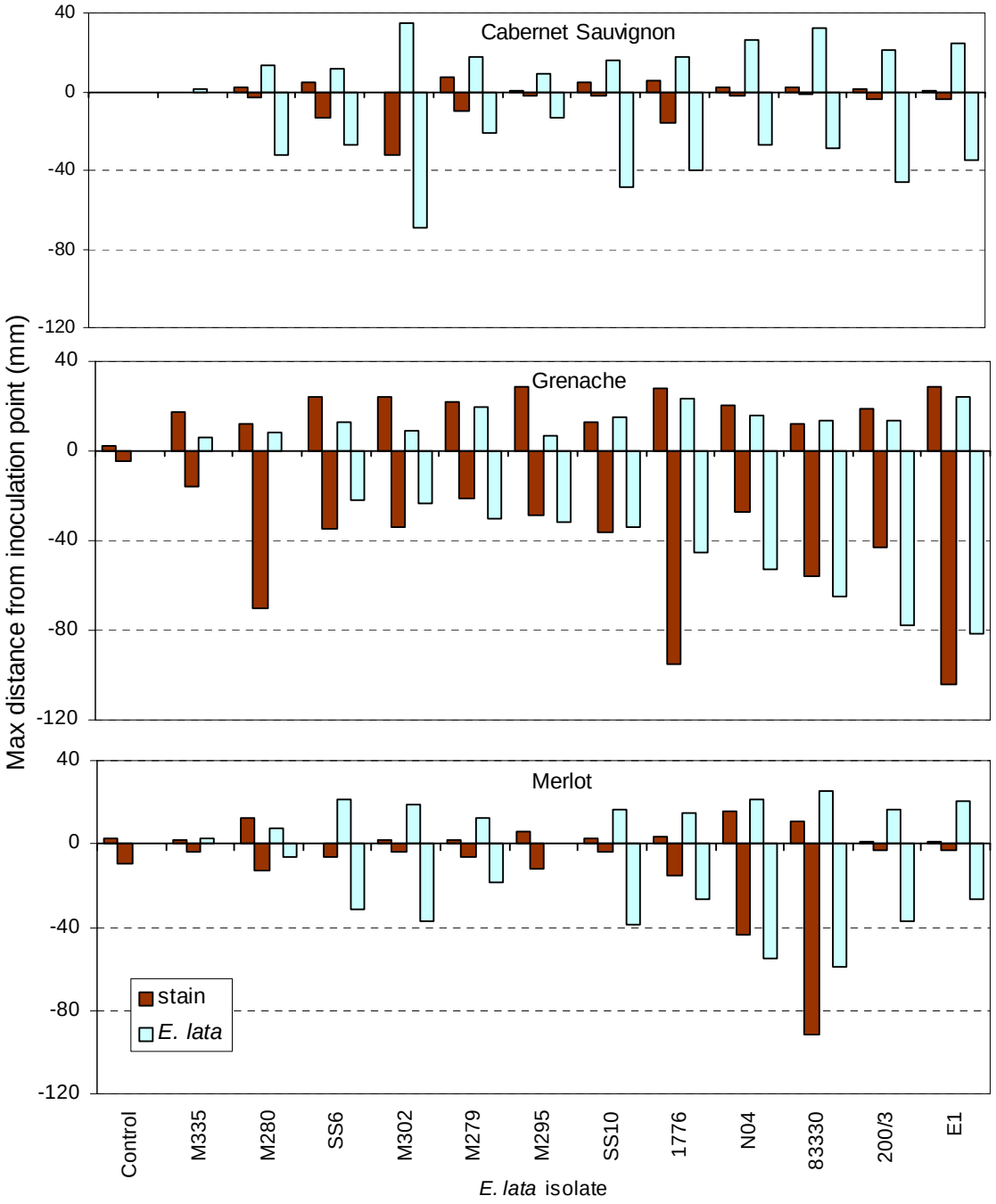


Figure 6.14. Extent of staining and colonisation by *E. lata* above and below (-) the inoculation site (0) on stems of three grapevine cultivars in Experiment 2 in the shadehouse, 20 months after inoculation with *E. lata*. Controls were inoculated with a blank agar plug.

The shadehouse bioassay showed that foliar symptoms of eutypa dieback can be induced on rooted vines within 8 months of inoculation. The initial results confirm reports by Péros and Berger (1994) suggesting that isolates of *E. lata* vary in their ability to induce foliar symptoms of eutypa dieback on grapevines. The preliminary observation that Grenache was more susceptible than the other cultivars tested also confirms previous research (Carter, 1991; Highet and Wicks, 1998). Geographical origin of isolates influenced the severity of foliar symptoms but no such effect was observed for colonisation by *E. lata*. The appearance of symptoms on shoots arising from the stem below the inoculation site suggested that toxic compounds may be

translocated in both directions in the wood, or that mycelium has colonised the wood below the inoculation point much more rapidly than expected. Both suggestions may be valid, as the fungus was detected from 30 to 195 mm below the inoculation point in these cuttings. This observation may be an artefact of the experimental conditions, however if the effect is real, there may be implications for disease management.

Over the 4 years of the field trial on mature vines, foliar symptoms were not observed on any vines, which is consistent with past field studies reporting that the delay between infection and symptom expression may be up to 8 years (Tey–Rulh *et al.*, 1991). In comparison, in the shadehouse, foliar symptoms were induced on rooted cuttings within 8 months of inoculation. This may be because the mycelial inoculum used in the shadehouse grew faster than the ascospore inoculum used in the field. Alternatively, the young vines may express symptoms more readily than mature vines, or the environmental conditions in the shadehouse may have been more conducive to symptom expression than those in the field. Péros and Berger (1994) reported characteristic symptoms within 4 weeks of inoculation of non–rooted cuttings. The variation in symptom severity seen 20 months after inoculation confirmed the report by Péros and Berger (1994) that isolates of *E. lata* vary in their ability to cause disease. Grenache displayed considerably more severe foliar symptoms than Merlot or Cabernet Sauvignon in the shadehouse, supporting previous reports that Grenache is less tolerant of eutypa dieback than these cultivars (Carter, 1991; Highet and Wicks, 1998). However, the shadehouse study suggests that Cabernet Sauvignon has a tolerance similar to Merlot, which is not consistent with past reports or with field observations. It is possible that the differences observed in susceptibility among cultivars in the shadehouse may, in part, reflect the immaturity of the tissue, which was 18 months old when inoculated. As the wood ages, differences in susceptibility between Cabernet Sauvignon and Merlot may become more apparent.

Growth rate of *E. lata* in Grenache cuttings in the shadehouse varied significantly among isolates and, although there was a correlation between geographic origin and foliar symptom expression, there was no such correlation with spread of the fungus. The reason for the first correlation may be that isolates are better suited to the climate in the region than the climate at the trial site. Furthermore, there was no correlation between the growth rate of *E. lata* and its ability to induce foliar symptoms. Susceptibility to expression of foliar symptoms appears to correlate with wood staining but not with the growth rate of the fungus in infected tissue. This suggests that wood staining may, in part, be caused by factors other than *E. lata*, such as a wound response. This was supported by the observation that control vines, wounded and treated with blank agar plugs, also exhibited some staining, as did vines inoculated with other species of wood–inhabiting fungi including *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, *Botryosphaeria* sp. *Libertella* sp. and *Fomitiporia* sp. Alternatively, the correlation between expression of foliar symptoms and vascular staining may be because isolates which induce foliar symptoms also produce greater amounts of hydrolytic enzymes which cause the degradation and staining of woody tissue.

The maximum distance to which *E. lata* extended ahead of stained wood in the shadehouse experiment was 80 mm, which may be the ‘worst case scenario’ since growth rate of the fungus in mature vines is slower. This means that when reworking, growers should cut at least 10 cm beyond the last sign of staining to ensure removal of the fungus, confirming the current recommendation of cutting 10–20 cm beyond stained tissue (Creaser and Wicks, 2002). These data represent 2 years of fungal growth and it is important to determine if the distance between discolouration and fungus might change over longer periods, such as 5–10 years. Mycelium of *E. lata* spread more rapidly down than up the stem in the shadehouse, however, it was interesting to note that mycelium could grow upwards in vines as it is generally considered that mycelium spreads down the vine only. There are no data to confirm if the pathogen grows in both directions in mature vines, and this should be investigated as it may have implications for managing the disease.

Staining attributed to necrosis caused by colonisation of wood by *E. lata* progressed at 10–18 mm/yr in mature field vines (section 5.1.2). When compared with the spread of staining on cuttings in the shadehouse, Merlot was similar at 8 mm/yr but, in contrast to the field trial results, Cabernet Sauvignon and Grenache progressed at 4 and 22 mm/yr, respectively. This may reflect the small sample size of eight vines of Grenache compared to 16 to 80 vines for the other cultivars in the field. Also cultivars may react differently to colonisation by *E. lata* in wood of younger cuttings compared with more mature vines in the field. Alternatively, the observation that Grenache, generally considered susceptible to eutypa dieback, appeared resistant to staining whereas Pinot Noir (generally considered tolerant) appeared less resistant, may provide further evidence that the ability of *E. lata* to induce foliar symptoms is not linked to its ability to colonise the wood.

6.1.5 Survey of Hunter Valley and Mudgee, New South Wales

Many vines were found with dead spurs or cordons (Figure 6.15A–B) which, when cut in cross–section showed wedges of discoloured tissue (Figure 6.15C–E) similar to wood symptoms associated with infection by *E. lata* (Figure 5.1). However, the characteristic foliar symptoms of eutypa dieback were not observed in any of the vineyards inspected. Furthermore, *E. lata* was not isolated from any of the wood samples taken from the two regions. However, some fungi were recovered and preliminary identification suggests that *Botryosphaeria* may be associated with dead wood but further testing is required to identify the species (S. Savocchia, personal communication 2003).

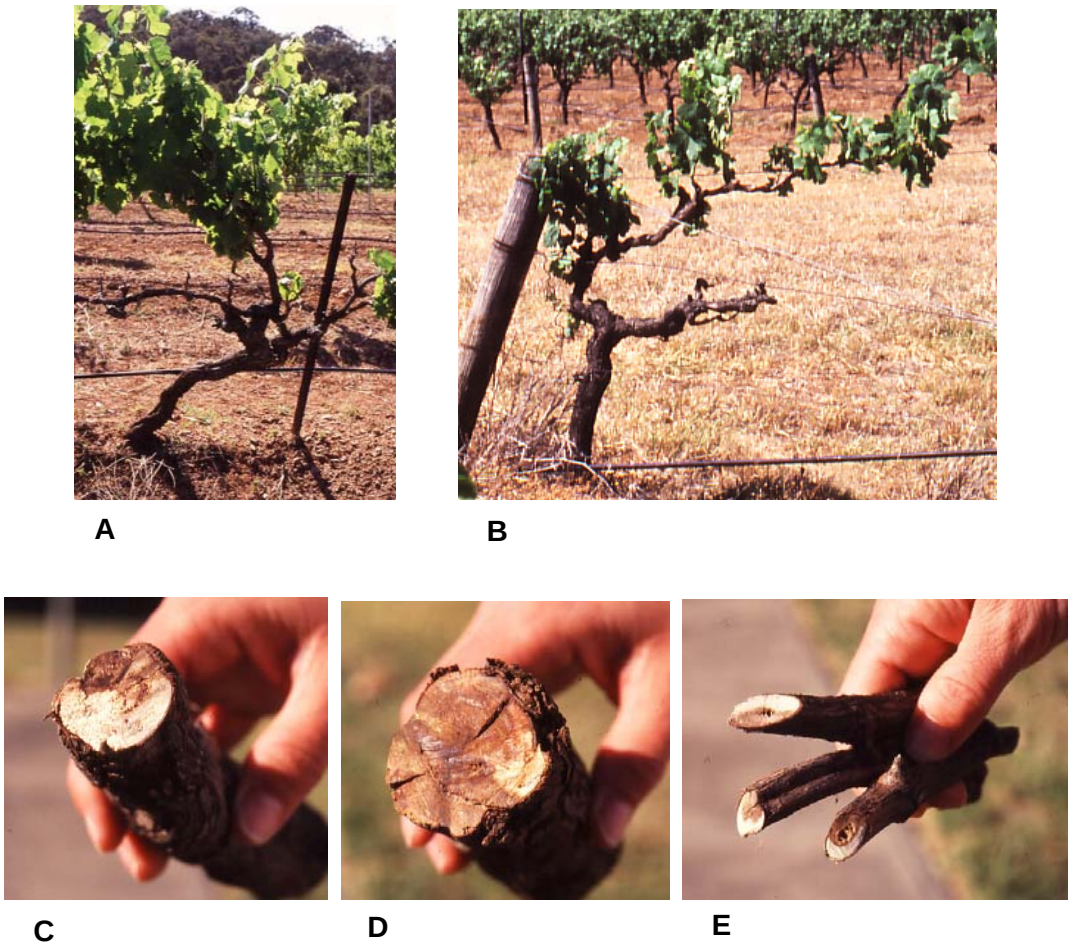


Figure 6.15. (A,B) Shiraz vines with dead spurs and cordons in the Hunter Valley, NSW. (C–E) Wedges of dead discoloured tissue observed in the Hunter Valley when dead spurs or cordons were cut in cross–section.

Despite the frequent occurrence of eutypa dieback in South Australia (Wicks, 1975; Highet and Wicks, 1998), a survey of 20 vineyards has revealed that the pathogen may be absent from the Hunter Valley and Mudgee wine regions. In the USA, there is a climatic divide between the occurrence of *E. lata* and *Botryosphaeria* spp. (Leavitt, 2003). A similar climatic divide may separate the occurrence of these trunk pathogens in Australia. The Hunter Valley has a warm to hot, sub–tropical climate whereas South Australia has a Mediterranean climate with hot dry summers and wet winters.

6.2 Prevention

6.2.1 Evaluation of treatments for the prevention of wound infection

6.2.1.1 Laboratory evaluation

Experiments 1 and 2. Using a series of concentrations, PDA was amended with Benlate (0–20 g/L), Flint (0–25 g/L) and Nustar (0–8 g/L). Benlate and Flint did not inhibit germination of *E. lata* ascospores at any of the rates tested, compared to the control with 0 g/L, but Nustar prevented germination at rates of 0.04 g/L and above. At rates of 0.31 g/L and above Benlate prevented mycelial growth and, at all rates tested, Flint inhibited mycelial growth by 24–55%. PDA amended with Nustar prevented mycelial growth at rates of 1.56 g/L and above, and as the rates decreased to 0.004 g/L the inhibition decreased to 33%.

Experiment 3. PDA was amended with Amistar, Benlate, Flint, Nustar, Ridomil Gold, Rovral, Topas and Ziram at 1 and 10 ppm. Nustar and Topas prevented ascospore germination at both 1 and 10 ppm, whereas Ridomil Gold only prevented germination at 10 ppm. No other fungicide tested inhibited ascospore germination. Benlate, Nustar and Topas prevented mycelial growth at 1 and 10 ppm. Ridomil Gold, Rovral and Ziram inhibited mycelial growth by 54–78% at 10 ppm compared to the control and inhibition was significantly reduced for these fungicides at 1 ppm, together with all other fungicides tested at both concentrations.

Experiment 4. PDA was amended with Benlate, Bravo, Dithane, Nustar, Octave, Shirlan and Topas at 1 and 10 ppm. Shirlan, Octave and Bravo prevented ascospore germination at both 1 and 10 ppm and Topaz, Nustar and Dithane prevented germination at 10 ppm only. Benlate did not inhibit germination of ascospores at either concentration. Benlate, Nustar, Shirlan and Topas prevented mycelial growth at 1 and 10 ppm and Octave prevented mycelial growth at 10 ppm and inhibited mycelial growth by 79% at 1 ppm compared to the control. Bravo and Dithane inhibited mycelial growth by 24–52% at both concentrations.

Experiment 5. PDA was amended with the treatments listed in Table 6.3 at a single concentration of 1 ppm. Bravo, Octave, Scala and Shirlan prevented ascospore germination and Nustar and Topas inhibited germination by 80% compared to the control. No other treatment inhibited ascospore germination. Benlate, Fungaflor, Nustar, Shirlan and Topas inhibited mycelial growth by 96–100%, Octave inhibited mycelial growth by significantly less (79%) and all other treatments inhibited mycelial growth by 48% or less. Citowett and Kocide had no inhibitory effect on mycelial growth of *E. lata*.

Experiment 6. PDA was amended with the treatments listed in Table 6.4 at a single concentration of 1 ppm. BASF516 and Cabrio prevented ascospore germination, Bayfidan inhibited germination by 94% and Teldor and Thiovit did not inhibit germination. BASF516, Bayfidan and Cabrio inhibited mycelial growth by 42–50% compared to the control but Teldor and Thiovit only inhibited mycelial growth by 2–7%.

Laboratory assessment of a range of products, which are registered for use against various pathogens on grapevines, showed that several products had the potential to control *E. lata*. Shirlan, Octave, Scala, Bravo, Topas and Nustar provided 80–100% inhibition of ascospore germination. Of these, Shirlan, Octave, Topas, Scala and Nustar also inhibited mycelial growth by 50–100% and, hence, were chosen for field evaluation. The remainder of the products assessed did not inhibit germination, but Fungaflor and Benlate provided 97–100% inhibition of mycelial growth so were also included in the field evaluation.

Table 6.3. Efficacy of treatments at 1 ppm for control of *E. lata* (Experiment 5). Treatment efficacy was evaluated based on the mean percent germination of 400 ascospores and mean diameter of mycelial growth from a 5 mm plug on fungicide amended PDA with ten replications. Cultures were measured after 7 days of incubation. Percentage of inhibition was calculated for treatments based on the germination percentage and culture diameter of unamended control plates.

Treatment	Germination %	Inhibition of germination %	Colony diameter mm	Inhibition of colony growth %
Shirlan	0^{c}	100	2^{h}	97
Octave	0^{c}	100	11^{g}	79
Scala	0^{c}	100	26^{f}	48
Bravo	0^{c}	100	41^{d}	21
Topas	20^{b}	80	1^{h}	98
Nustar	20^{b}	80	2^{h}	96
Fungaflor	100^{a}	0	0.1^{h}	100
Benlate	100^{a}	0	2^{h}	97
Flint	100^{a}	0	32^{e}	38
Amistar	100^{a}	0	36^{d}	29
Ziram	100^{a}	0	37^{d}	27
Ridomil Gold	100^{a}	0	42^{cd}	17
Rovral	100^{a}	0	46^{c}	11
Dithane	100^{a}	0	51^{b}	1
Control	100^{a}	0	51^{b}	0
Citowett	100^{a}	0	56^{ab}	0
Kocide	100^{a}	0	60^{a}	0

Values within a column with the same superscript are not significantly different (P = 0.05)

Table 6.4. Efficacy of treatments at 1 ppm for control of *E. lata* (Experiment 6). Treatment efficacy was evaluated based on the mean percent germination of 400 ascospores and mean diameter of mycelial growth from a 5 mm plug on fungicide amended PDA with five replications. Cultures were measured after 3 days of incubation. Percentage of inhibition was calculated for treatments based on the germination percentage and culture diameter of unamended control plates.

Treatment	Germination %	Inhibition of germination %	Colony diameter mm	Inhibition of colony growth %
Cabrio	0^{c}	100	10^{d}	50
BASF516	0^{c}	100	11^{c}	42
Bayfidan	6^{d}	94	10^{cd}	47
Teldor	100^{a}	0	17^{b}	7
Thiovit	100^{a}	0	18^{a}	2
Control	100^{a}	0	18^{a}	0

Values within a column with the same superscript are not significantly different (P = 0.05)

6.2.1.2 Field evaluation

Trial 1. Application of Fungaflor, Hayright, Shirlan, Scala, Paint + Benlate, Nectec, Benlate, Garrison, ATCS paint and Solucuivre to pruning wounds all significantly reduced the MPR of *E. lata* when inoculated 1 day after treatment, with no *E. lata* recovered from canes treated with the latter four treatments (Figure 6.16). When inoculation occurred 14 days after treatment, Paint + Benlate, Nectec, Benlate and ATCS paint were the only treatments to reduce MPR of *E. lata* significantly.

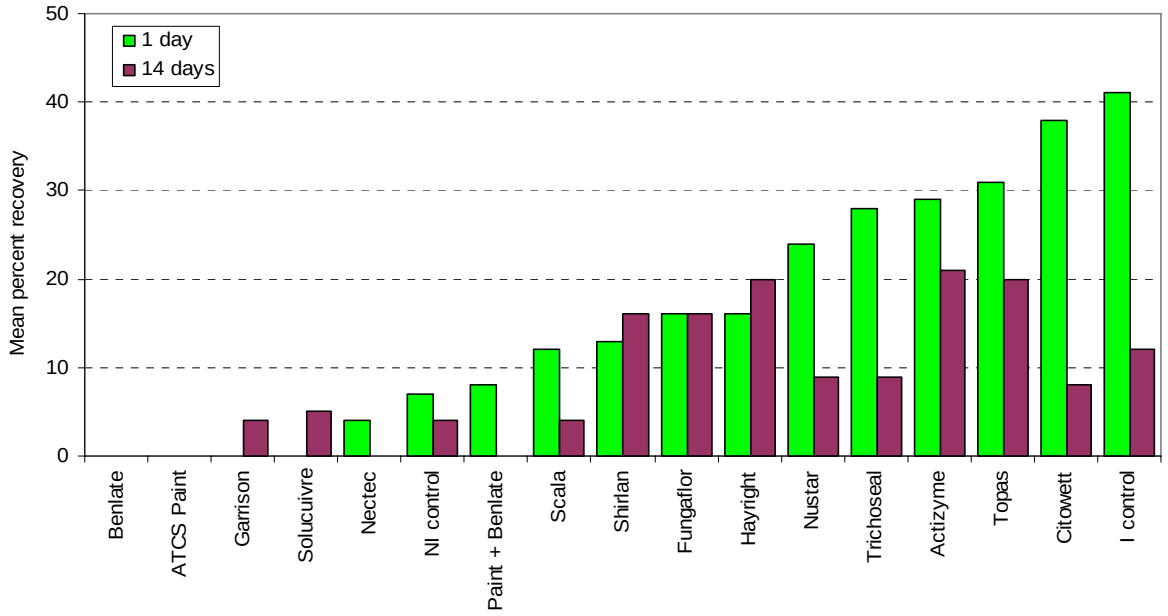


Figure 6.16. Efficacy of treatments for control of *E. lata* (Trial 1). Inoculation with 500 ascospores/wound occurred 1 and 14 days after treatment. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ (1 day) = 10, $LSD_{0.05}$ (14 days) = 20.

Trial 2. MPR of *E. lata* was not significantly reduced by any treatment compared to the control when inoculated 1 day after treatment, although *E. lata* was not recovered from spurs treated with Benlate, Garrison, ATCS paint and Solucuivre (Figure 6.17). *E. lata* was not recovered from controls inoculated 14 days after treatment, so data are not shown.

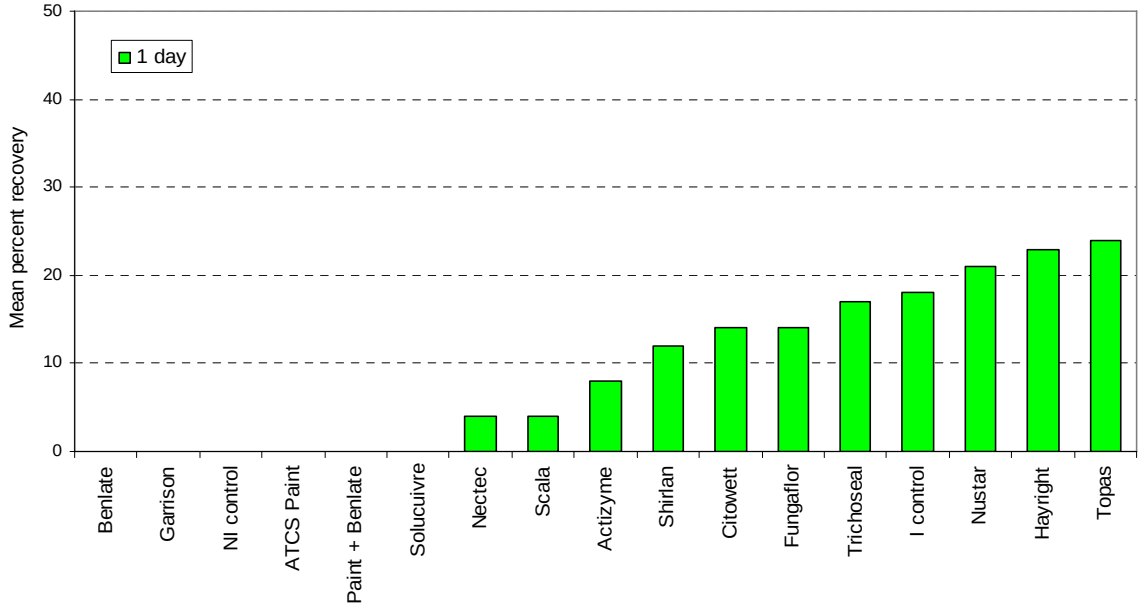


Figure 6.17. Efficacy of treatments for control of *E. lata* (Trial 2). Inoculation with 500 ascospores/wound occurred 1 day after treatment. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ (1 day) = 18.

Trial 3. *E. lata* was not recovered from spurs treated with Benlate, ATCS paint, Paint + Benlate and Fungaflor, when inoculation occurred 1 day after treatment, representing a significant reduction in MPR of *E. lata* (Figure 6.18). When inoculated 14 days after treatment, MPR of *E. lata* was significantly reduced by applications of Citowett, Actizyme, Nectec, Garrison, Solucuivre, Benlate, ATCS paint and Paint + Benlate, with all but Actizyme preventing colonisation of the wounds.

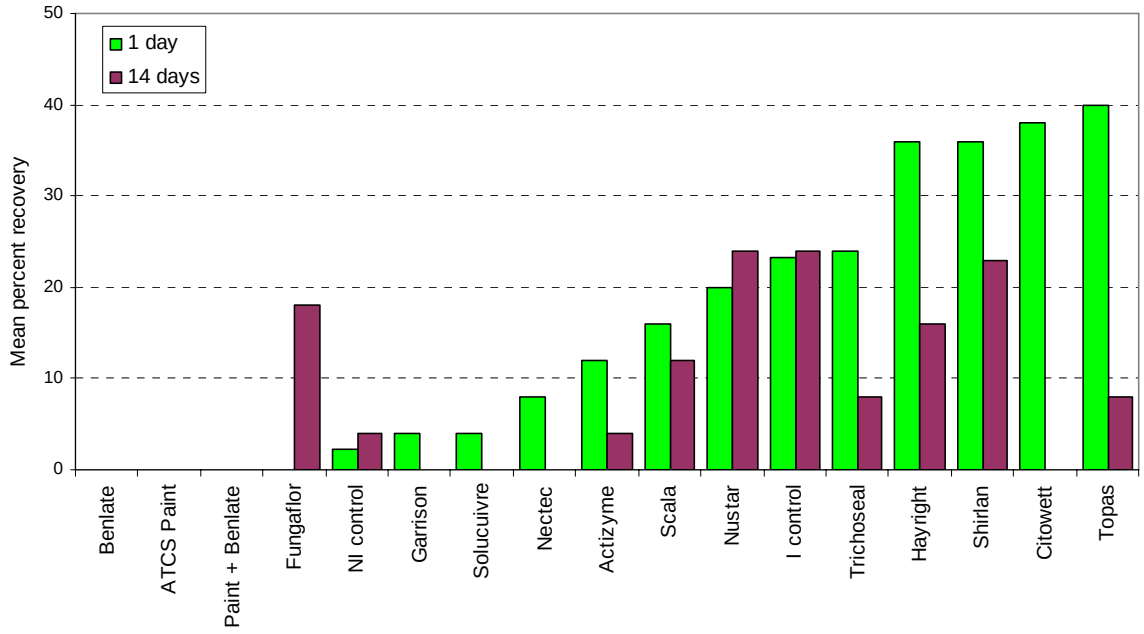


Figure 6.18. Efficacy of treatments for control of *E. lata* (Trial 3). Inoculation with 500 ascospores/wound occurred 1 and 14 days after treatment. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ (1 day) = 22, $LSD_{0.05}$ (14 days) = 18.

Trial 4. Application of Scala, Solucuivre and Bavistin significantly reduced the MPR of *E. lata* as both preventative and curative treatments (Figure 6.19). *E. lata* was not recovered from spurs treated with Bavistin. Fungaflor significantly reduced the MPR of *E. lata* as a curative treatment.

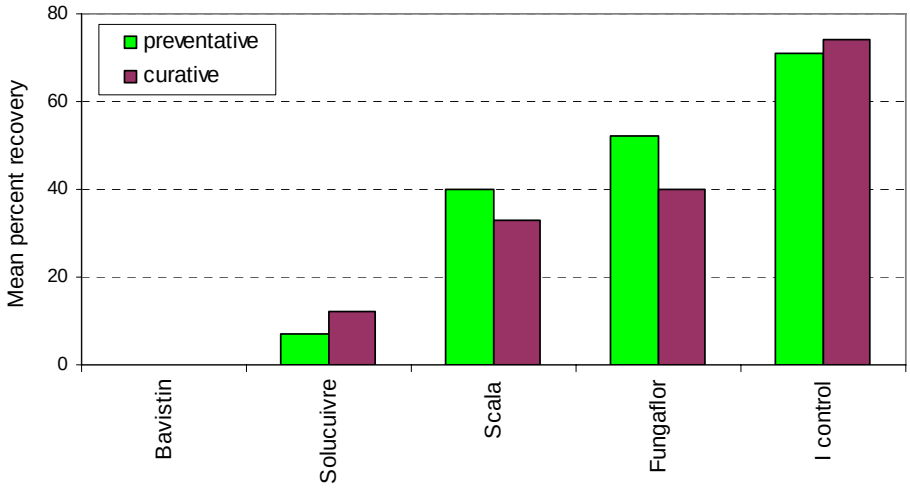


Figure 6.19. Efficacy of treatments for control of *E. lata* (Trial 4). Inoculation with 500 ascospores/wound occurred 1 day after treatment (preventative) and 1 day before treatment (curative). Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ (preventative) = 27, $LSD_{0.05}$ (curative) = 32.

Trial 5. *E. lata* was not recovered from spurs treated with Bavistin (12.5 g a.i./L water) when inoculation occurred 1 and 14 days after treatment, representing a significant reduction in MPR of *E. lata* (Figure 6.20). At the lower rates of 6.25 and 3.13 g a.i./L, MPR of *E. lata* was reduced when inoculation occurred 1 and 14 days after treatment, however the reduction was only significant in the case of inoculation at 14 days, where *E. lata* was not recovered from treated canes.

E. lata did not colonise wounds treated with Fungaflor at 6.25 g a.i./L when inoculated 14 days after treatment. Reduction in MPR of *E. lata* was not significant at other rates of Fungaflor.

Nustar (12.5 g a.i./L water) significantly reduced the MPR of *E. lata* by 85–86% when inoculated at 1 and 14 days. At the lower rates of 6.25 g and 3.13 g a.i./L, MPR of *E. lata* was reduced by 41–78% when inoculated 1 and 14 days after treatment.

Shirlan significantly reduced the MPR of *E. lata* by 86% at 12.5 g a.i./L when inoculated 1 day after treatment and *E. lata* was not recovered from treated canes at 6.25 g a.i./L when inoculated 14 days after treatment. Reduction in MPR of *E. lata* was not significant at other rates of Shirlan.

E. lata did not colonise wounds treated with Topas (12.5 g a.i./L water) when inoculation occurred at 1 or 14 days after treatment. At the lower rates of 6.25 g and 3.13 g a.i./L, MPR of *E. lata* was reduced by 55–86% and 70–85% when inoculated 1 and 14 days after treatment, respectively.

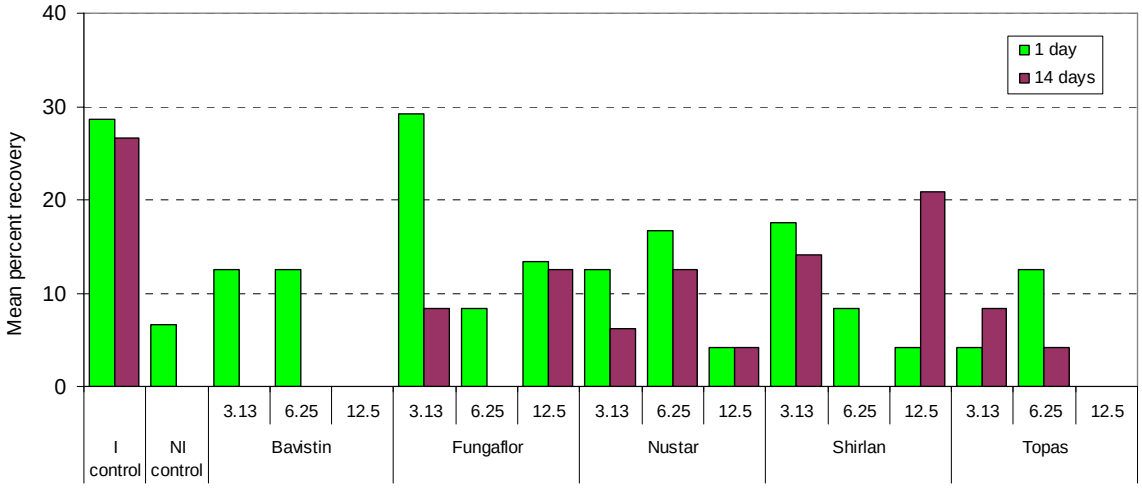


Figure 6.20. Efficacy of treatments for control of *E. lata* (Trial 5). Inoculation with 500 ascospores/wound occurred 1 and 14 days after treatment at rates of 3.13, 6.25 and 12.5 g a.i./L. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ (1 day) = 21, $LSD_{0.05}$ (14 days) = 18.

Trial 6. When inoculation occurred 1 day before treatment, Benlate, Bayfidan, Cabrio and BASF 516 reduced infection by *E. lata* significantly compared to the water control (Figure 6.21). When inoculation occurred 1 day after treatment, Benlate was the only treatment which reduced infection significantly and BASF516 and Cabrio had a smaller effect in reducing infection. Infection of wounds inoculated 2 weeks after treatment was reduced by Benlate only.

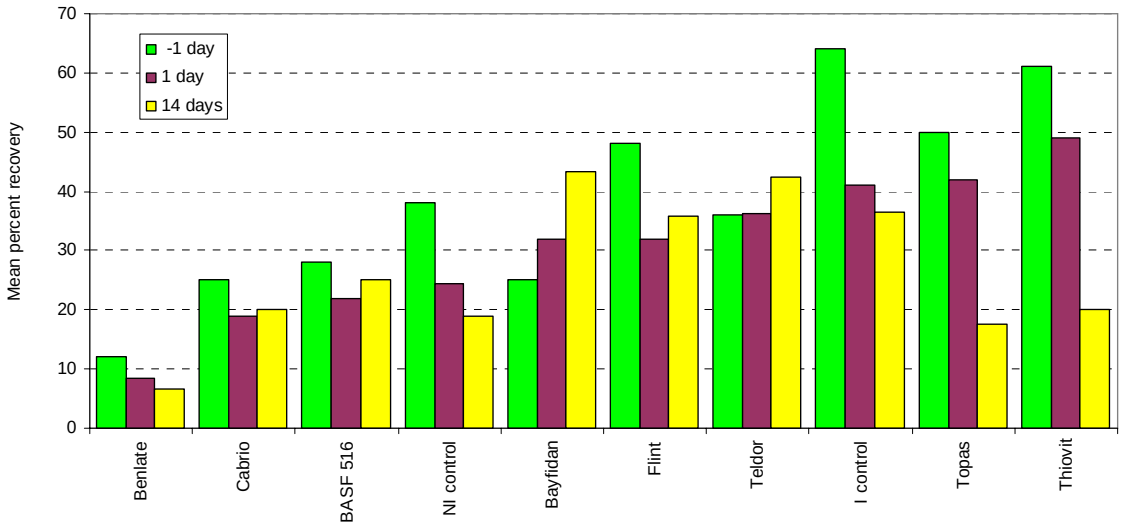


Figure 6.21. Efficacy of treatments for control of *E. lata* (Trial 6). Inoculation with 500 ascospores/wound occurred one day before (–1 day), one day after (1 day) and 14 days after (14 days) treatment. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ (–1 day) = 28, $LSD_{0.05}$ (1 day) = 17, $LSD_{0.05}$ (14 days) = 23.

When the same trial was analysed based on detection of *E. lata* DNA in wood by the slot blot assay, results differred somewhat from those obtained following isolation into culture (compare Figure 6.21 and Figure 6.22). For example, MPR from the NI controls were greater following isolation than 'MPR' using the slot blot analysis of DNA. This suggests that natural infection was rare and that some of the fungi isolated on agar were *Eutypa*–like fungi rather than *E. lata*. Statistical comparison of the results obtained by isolation and by slot blot analysis, shown in Figure 6.23 illustrates this inconsistency, with data points on either side of the line representing treatments for which the two methods provided different results

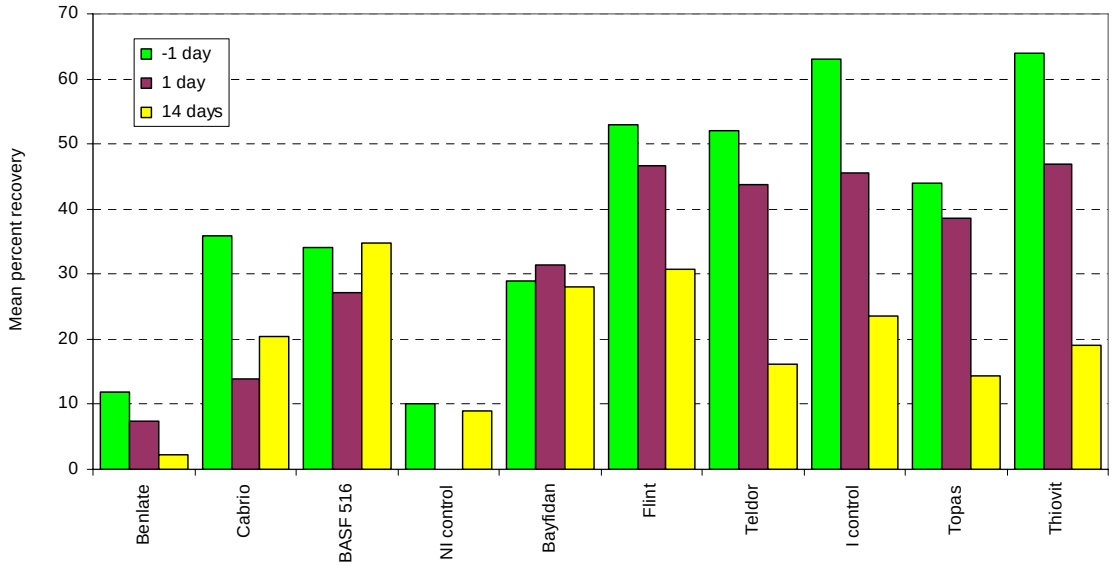


Figure 6.22. Efficacy of treatments for control of *E. lata* (Trial 6). Inoculation with 500 ascospores/wound occurred one day before (–1 day), one day after (1 day) and 14 days after (14 days) treatment. Treatment efficacy was determined by the MPR of *E. lata* from treated spurs using slot blot analysis of DNA extracted from wood. Controls, I and NI, were treated with SWD. $LSD_{0.05}$ (–1 day) = 30, $LSD_{0.05}$ (1 day) = 19, $LSD_{0.05}$ (14 days) = 15.

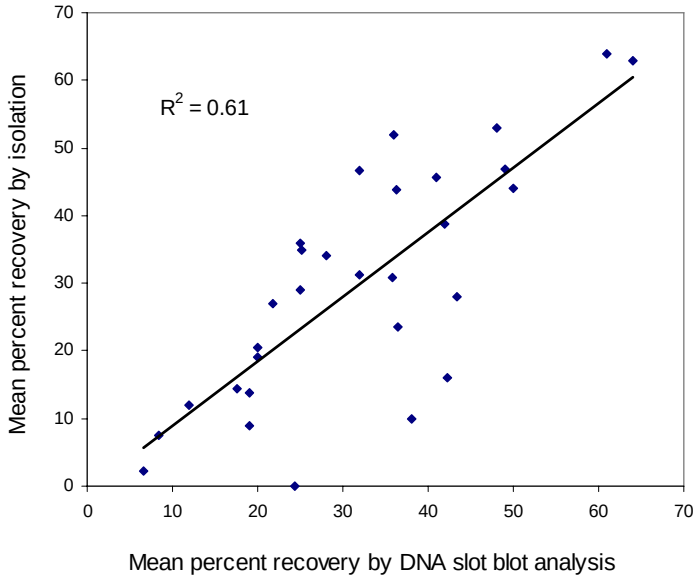


Figure 6.23. Relationship between MPR data from isolation of *E. lata* on PDA and slot blot analysis of DNA extracted from wood in Trial 6 to evaluate the efficacy of treatments applied for the control of *E. lata* when inoculated with 500 ascospores/wound.

Trial 7. MPR was very low for the inoculated control (approximately 4%) when assessed by isolating *E. lata* and, hence, the results for all treatments were not significantly different from each other (Figure 6.24). However, results from slot blot analysis of DNA extracted from the same samples showed that the MPR of the inoculated control was 40% and all treatments significantly reduced MPR compared to the inoculated control. Contamination of plates by a *Libertella* sp., which had similar morphology to *E. lata* and was mistakenly used to inoculate wounds initially, contributed to the low MPR of *E. lata* isolated from canes on PDA. The DNA slot blot method was able to detect *E. lata* present as a result of the second inoculation with *E. lata*, 6 days after treatment.

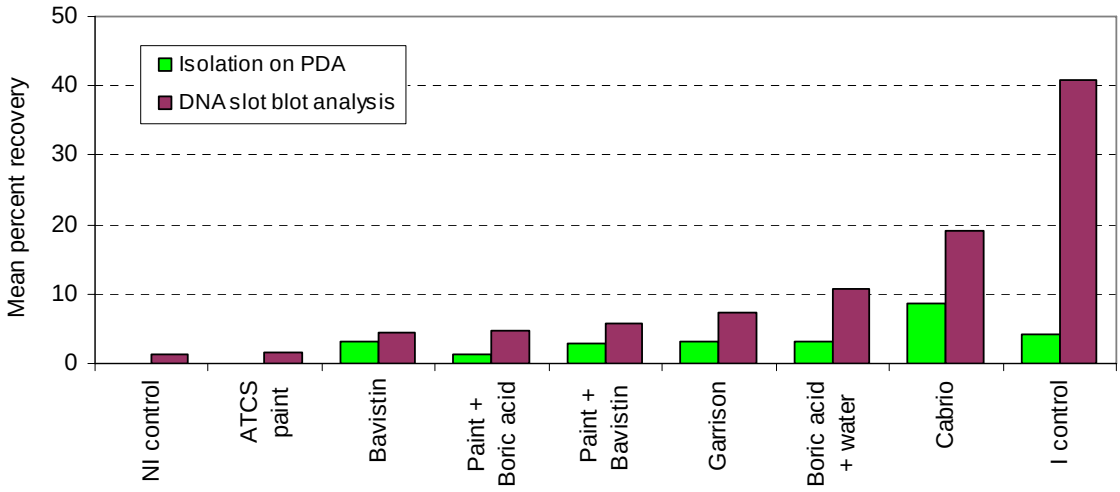


Figure 6.24. Efficacy of treatments for control of *E. lata* (Trial 7). Inoculation with 1000 ascospores/wound occurred 6 days after treatments were applied. Treatment efficacy was determined based on the MPR of *E. lata* isolated and by slot blot analysis of DNA extracted from wood of treated canes. Controls, I and NI, were treated with SDW. $LSD_{0.05}$(MPR) =4.9, $LSD_{0.05}$(DNA) = 9.8.

The susceptibility of wounds to infection by *E. lata* decreased with time. The pathogen was recovered from more canes when inoculated the day following wounding than from those inoculated 14 days later. This is evident in the inoculated controls and variation may be caused by weather factors which influence ascospore production, germination and mycelial growth in the wood.

Of the fungicides evaluated, Benlate (at 2 or 25 g/L) was the most effective in preventing infection by *E. lata* and provided both immediate and long–lasting protection. However, the manufacture of Benlate and other benomyl products has been discontinued.

An alternative benzimidazole fungicide, Bavistin (carbendazim), however, is already registered for the control of other diseases on grapevine. In four separate trials, when treated wounds were challenged with ascospores of *E. lata* 1, 7 or 14 days after treatment, infection was inhibited when Bavistin was applied at 6.25–25 g/L. Furthermore, when Bavistin was applied 1 day after infection by *E. lata*, it also inhibited establishment of *E. lata in* the wood. Solucuivre, a French product containing copper and carbendazim, also substantially inhibited infection by *E. lata*. These trials provide evidence for the efficacy of carbendazim as an alternative to benomyl for protecting pruning wounds from infection by *E. lata.*

Cabrio (pyraclostrobin), BASF 516 (boscalid + pyraclostrobin), Nustar (flusilazole), Shirlan (fluazinam), Scala (pyrimethanil) and Fungaflor (imazalil) were able to reduce infection by *E. lata*, although they were not as effective as carbendazim. All of these products are registered for use on grapevines, but for the control of diseases other than eutypa dieback. In comparison, research on pruning wound protection was presented at the 4th International Workshop on Grapevine Trunk Diseases held in Stellenbosch, South Africa in Jan 2005. In South Africa, flusilazole (Nustar) was as effective as benomyl, in Greece, fluazinam (Shirlan) and carbendazim provided 100% control of infection by *E. lata*, and in France, eutypa dieback control is based solely on a flusilazole and carbendazim mixture. All of these products are registered for use on grapevines in Australia, but for the control of diseases other than eutypa dieback.

Physical barriers such as paints and pastes also proved effective in limiting infection of pruning wounds. ATCS tree wound acrylic paint prevented infection through pruning wounds on 1–year old canes. Garrison and Nectec pastes, which also contain fungicides, substantially inhibited infection.

Biocontrol products containing *Trichoderma* spp. (such as Trichoseal spray and Vinevax) did not prevent infection by *E. lata* in these trials. In contrast, John *et al.* (2005b) recently reported the potential of *Trichoderma* to control eutypa dieback, as have reports from South Africa (Halleen and Fourie, 2005) and Greece (Bourbos and Barbopoulou, 2005).

DNA slot blot analysis can increase detection of *E. lata* compared to assessment by isolation on PDA. Detection of the fungus by the slot blot was sometimes greater but also sometimes less than that by isolation in Trial 6. This may be due to contamination of some plates by wood inhabiting fungi and bacteria or by mis-identification of *E. lata*, which appears identical in culture to several other fungi. Nevertheless, each method led to the same conclusions about efficacy of treatments for control of *E. lata*. DNA slot blot analysis resulted in a greater recovery of *E. lata* compared to isolation on PDA in Trial 7. These findings highlight the importance of DNA technology to avoid problems such as contamination by other wood inhabiting fungi and for accurate conclusions on the efficacy of treatments for control of *E. lata*. Currently the protocol for DNA analysis using the *E. lata*–specific probes developed in this project is more costly and time–consuming than isolating *E. lata* on PDA. Further development is necessary so that this method, which can improve assessment sensitivity, may be utilised in future research for the management of eutypa dieback.

6.2.2 The effect of wound size on efficacy of wound treatments

Trial 1. Application of Paint + Bavistin, Garrison, ATCS Paint and Bavistin significantly reduced the MPR of *E. lata* by 65–85% on wounds 5–10 mm in diameter (Figure 6.25). MPR was not reduced when treatments were applied to larger wounds (20–30 mm diameter). Infection by *E. lata* in the inoculated controls was less for the larger wounds than for the small wounds in Trial 1. The reason for this is unclear, but lack of infection confounded the analysis of efficacy.

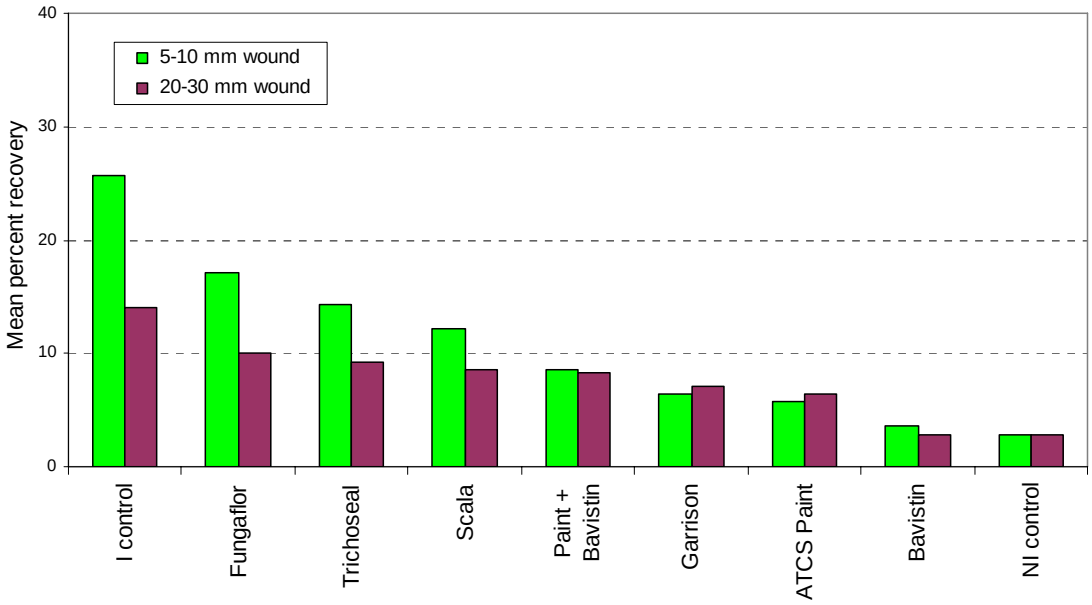


Figure 6.25. Efficacy of treatments for control of *E. lata* (Trial 1). Inoculation with 500 ascospores/wound occurred 1 day after treatment on wounds of different size (0.5–1 cm and 2–3 cm diameter). Treatment efficacy was determined based on the MPR of E. lata from the treated spurs. Controls, I and NI, were treated with SDW. LSD0.05 (0.5–1 cm) = 15, LSD0.05 (2–3 cm) = 15.

Trial 2. There was no significant effect of application of any of the treatments compared to the I control, which had a MPR of 34%. The reason for this is unclear but it may be that *E. lata* was already present in the wood tissue ahead of the discoloured tissue from infection occurring before the commencement of the trial.

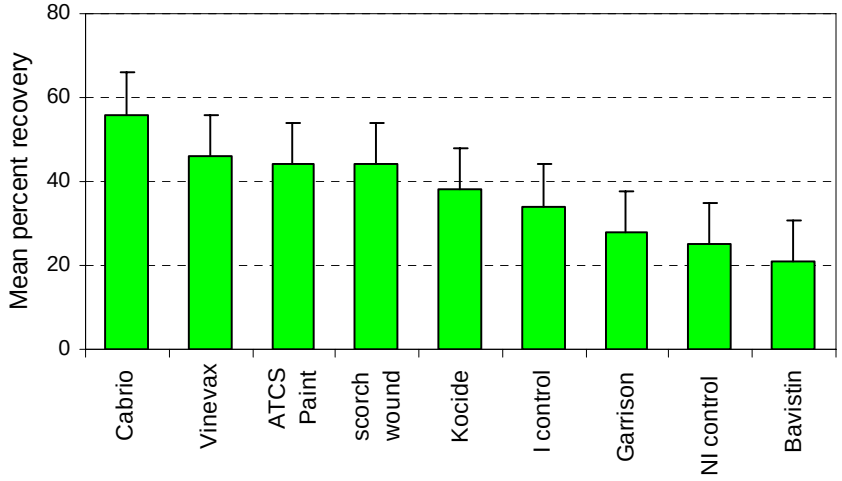


Figure 6.26. Efficacy of treatments for control of *E. lata* (Trial 2). Inoculation with 25,000 ascospores/mL water occurred 1 day after treatment on cordon wounds of diameter > 4 cm. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs using DNA slot-blot analysis. Controls, I and NI, were treated with SDW. Bars represent standard error of the mean.

6.2.3 The efficacy of spray application of wound treatments

Trial 1. There was no difference in MPR of *E. lata* between treatments applied with paint brush and a hand–held sprayer and so data were combined for statistical analysis. When inoculations were made 1 day after treatments were applied, Scala, Solucuivre and Bavistin significantly reduced the MPR of *E. lata* by 62–87% (Figure 6.27). When inoculation was 7 days after treatment, Solucuivre and Bavistin significantly reduced MPR of *E. lata* by 86–91%. When wounds were inoculated 14 days after treatment, MPR of *E. lata* was not reduced significantly, although the reduction was 82–100% for Solucuivre and Bavistin.

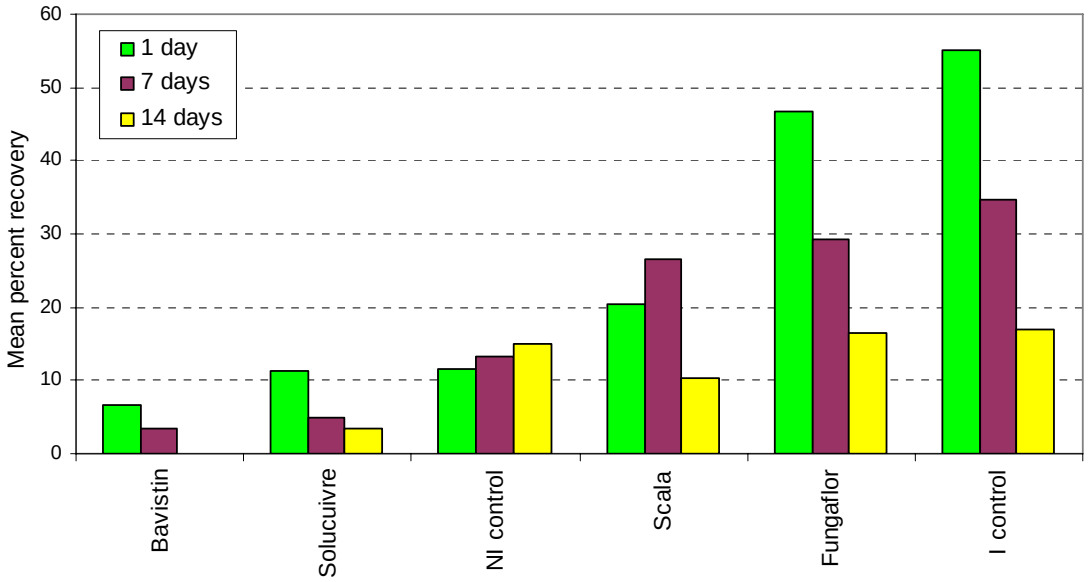


Figure 6.27. Efficacy of treatments for control of *E. lata* (Trial 1). Inoculation with 500 ascospores/wound occurred 1, 7 and 14 days after treatment, with data from application by hand–held sprayer and paint brush combined. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls (I and NI) were treated with SDW. $LSD_{0.05}$ (1 day) = 20, $LSD_{0.05}$ (7 days) = 15, $LSD_{0.05}$ (14 days) = 18.

Trial 2. Benlate applied as a spray significantly reduced MPR of *E. lata* compared to the I control when inoculation occurred 1 day after treatment, and was more effective than application with a paint brush in this trial (Figure 6.28). Neither treatment gave a significant reduction in MPR of *E. lata* compared to the I control when inoculation occurred 14 days after treatment.

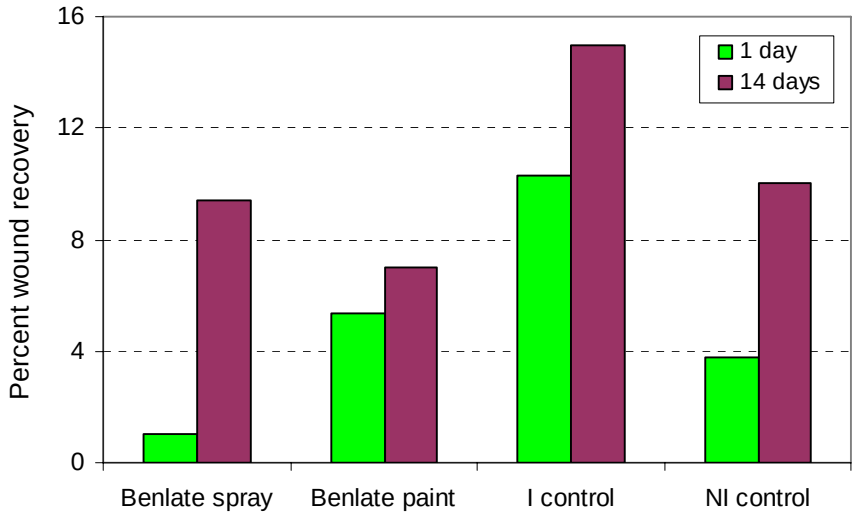


Figure 6.28. Comparison of Benlate applied as a spray or with a paint brush for control of *E. lata* (Trial 2). Inoculation with 500 ascospores/wound occurred 1 and 14 days after treatment. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ (1 day) = 7, $LSD_{0.05}$ (14 days) = 16.

Trial 3. Bavistin significantly reduced MPR of *E. lata* compared to the I control when applied with either the SARDI fan or the air–blast sprayer, and spray application was as effective as painting in this trial (Figure 6.29). The low MPR for the I control may be because of non–conducive weather conditions for germination and mycelial growth following inoculation or due to contamination of agar plates with *Libertella* sp., which was mistakenly used to inoculate the trial initially.

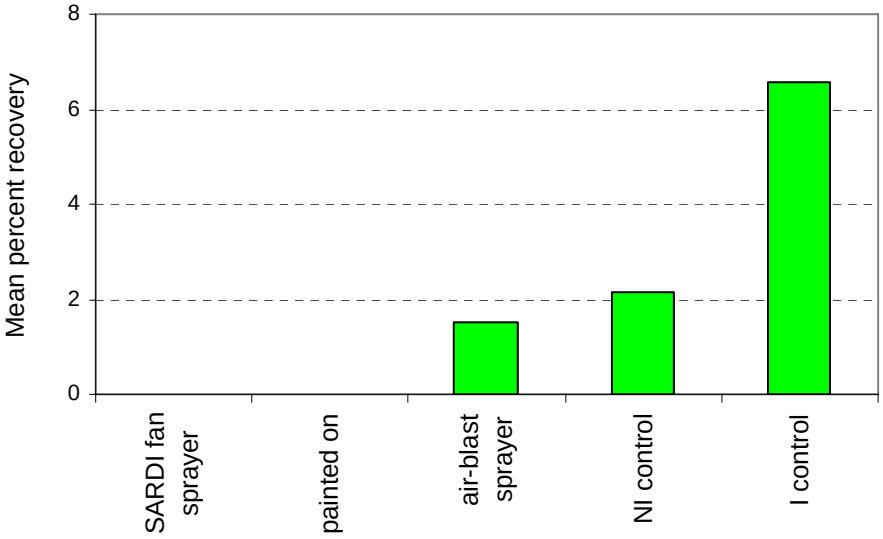


Figure 6.29. Comparison of Bavistin applied with commercial spray systems and a paint brush for control of *E. lata* (Trial 3). Inoculation with 1000 ascospores/wound occurred 14 days after treatment. Bavistin was applied either using a Hardi air–blast sprayer (310 L/ha), a SARDI fan sprayer (596 L/ha), or by hand with a paint brush. Treatment efficacy was determined based on the MPR of *E. lata* from the treated spurs. Controls, I and NI, were treated with SDW. $LSD_{0.05}$ = 4.4.

Preliminary results indicate that spray application of Benlate or Bavistin fungicides for protection of pruning wounds can reduce MPR compared to inoculated controls and, in these trials, was as effective as application with a paint brush. However, the use of commercial sprayers may be limited by factors such as spray drift and chemical residues in the soil, so further work is necessary to assess several spray systems and various rates to achieve optimal protection whilst minimising cost.

6.2.4 Residue analysis

6.2.4.1 Benlate (a.i. benomyl)

In both trials, carbendazim was not detected in any of the fruit from vines treated with benomyl (amounts below 0.01 mg/L are reported by the AWRI as "not detected").

6.2.4.2 *Garrison (a.i. cyproconazole)*

The AWRI reported that cyproconazole was not detected in any of the four samples tested.

6.3 Control

6.3.1 Remedial surgery

For each trial, the percentage of vines with watershoots and foliar symptoms of eutypa dieback is given for up to 6 years after remedial surgery (Table 6.5). Furthermore, for some trials, pre–surgery symptoms have also been recorded.

Foliar symptoms were visible on only 35% of Shiraz vines in Trial 1 in Eden Valley, yet all vines contained discoloured wood in the cordons. Furthermore, 71% of vines contained infected wood that could not be removed, i.e. that was visible on the stump surface 30–40 cm above the ground. These residual infections did not affect watershoot production, as 63% of vines produced watershoots in the first spring following surgery. Likewise, pruning wound treatment did not influence watershoot production. Five years later, in spring 2005, the incidence of watershoot production had increased to 80% and eight vines (5.8%) expressed foliar symptoms of eutypa dieback. Of the eight vines displaying foliar symptoms, five were observed to have wedge–shaped staining typical of eutypa dieback immediately after surgery.

In Trials 2 and 3 at McLaren Vale, significantly more Malbec vines (95%) produced watershoots than Shiraz vines (76%) when examined in spring 2001, and none of the vines exhibited foliar symptoms of eutypa dieback. By spring 2005, the incidence of watershoot production decreased by 1% for Malbec, but increased by 4% for Shiraz, and foliar symptoms of eutypa dieback were observed on 1% of Malbec and 8.4% of Shiraz vines. In Trial 4, 91% of Shiraz vines produced watershoots and foliar symptoms were not recorded in the first season. Four seasons later, watershoot production had decreased by 4% and foliar symptoms were observed in 3.6% of vines.

Foliar symptoms were expressed by 56% of Shiraz vines during the spring before remedial surgery was conducted in Trial 5. In the spring after surgery, 79% of vines produced watershoots and 4% of vines displayed foliar symptoms, all of which recorded foliar symptoms pre–surgery. Three seasons later, watershoot production had increased by 10% and foliar symptoms by 2%.

Assessment of cross–sections made through trunks revealed that 17, 37 and 36% of vines had wood–staining characteristic of infection by *E. lata* in Trials 6, 7 and 8, respectively. In the following spring, 55, 51 and 42% of vines produced watershoots, but no foliar symptoms were observed. In Trial 6, four seasons after surgery, the percentage of Cabernet Sauvignon vines with watershoots decreased by 3%, and 20 vines (11%) displayed foliar symptoms. Of the 20 vines with foliar symptoms, seven trunks retained a wedge of stained wood after surgery. However, of the 58 vines which had wood symptoms at surgery, 51 showed no signs of foliar symptoms during the following 3 years. Three seasons after surgery, the percentage of Shiraz vines with watershoots increased by 5 and 34% in Trials 7 and 8 respectively. Foliar symptoms were observed in 1.3% of vines in Trial 8, however, no symptoms were observed in Trial 7.

Pinot Noir vines in Trial 9 were all free of any foliar or wood symptoms pre–surgery. In the following spring all vines produced watershoots with no effect of wound treatment and no foliar symptoms observed. Three seasons later, 98% of vines had watershoots and foliar symptoms were still not evident. In Trial 10, 92 % of Shiraz vines had watershoots in the season after surgery but no foliar symptoms were observed. Three years after surgery, 1% more vines had watershoots and 8.1% of vines displayed foliar symptoms. The percentage of Shiraz vines with watershoots following surgery in Trial 11 was 68%, which increased a further 8% in the following season. Foliar symptoms were not observed in the first season but 1.1% of vines displayed symptoms in the second season. In Trial 12, 61% of Cabernet Sauvignon vines produced watershoots in the season following surgery and this increased a further 17% in the following season. No foliar symptoms were recorded on vines over the two years of this trial.

Table 6.5. Summary of 12 remedial surgery trials conducted in five South Australian wine regions, on four cultivars of grapevine, including the year vines were planted and the number of vines in each trial. Vines were either cut through the trunk 30–40 cm above the ground using the cut and train (C&T) method or cordons were removed at the crown using the train and cut (T&C) method of remedial surgery. The percentage of vines with eutypa dieback symptoms was assessed in some trials pre–surgery (for foliar symptoms) and during surgery (for wood symptoms). All trials were assessed each spring following surgery for watershoot production and eutypa dieback symptoms in new foliage.

Trial	Region	Cultivar	Year planted	No. of vines	Method	Year of surgery	Symptoms pre–surgery (%)		Watershoots (%) Years (y) after surgery						Foliar symptoms (%) Years (y) after surgery					
							Foliar	Wood	1 y	2 y	3 y	4 y	5 y	6y	1 y	2 y	3 y	4 y	5 y	6y
1	EV	Shiraz	1971	141	C&T	2000	35	71	65	62	75	76	77	80	0	0	0	1.4	5.8	5.8
2	MV	Malbec	1970	871	C&T	2000	–	–	95	95	94	94	94	94	0	0	0	0.1	0.7	1.0
3	MV	Shiraz	1970	1135	C&T	2000	–	–	76	76	76	83	82	80	0	0	0	1.1	5.1	8.4
4	CW	Shiraz	1967	200	T&C	2002	–	–	91	87	87	87			0	0	0	3.6		
5	CW	Shiraz	1970	144	T&C	2003	56	–	79	89	89				4.0	1.0	6.0			
6	MV	Cab Sauv	1971	356	C&T	2002	–	17	55	52	53	52			0	0	4.0	11		
7	MV	Shiraz	1960	200	C&T	2003	–	37	51	57	56				0	0	0			
8	EV	Shiraz	1971	157	C&T	2003	–	36	42	76	76				0	0	1.3			
9	BV	Pinot Noir	1990	128	C&T	2003	0	0	100	98	98				0	0	0			
10	EV	Shiraz	1971	202	T&C	2003	–	–	92	92	93				0	0	8.1			
11	EV	Shiraz	1971	355	T&C	2004	–	–	68	76					0	1.1				
12	CL	Cab Sauv	1984	151	C&T	2004	–	0	61	78					0	0				

EV = Eden Valley, MV = McLaren Vale, CW = Coonawarra, BV = Barossa Valley and CL = Clare.

— = not assessed

When the vines in Trials 7 and 8 were cut, eutypa dieback symptoms were observed in the wood of 37% and 36% of vines, respectively, and wood symptoms characteristic of Petri disease and/or esca were observed in 49% and 93% of vines, respectively. Figure 6.30 shows a cross–section of a trunk with characteristic wood discolouration and indicates the locations from which the following fungi were isolated in this study; *E. lata* which causes eutypa dieback, *P. chlamydospora* and *P. aleophilum*, which have been associated with both Petri disease and esca (Edwards and Pascoe, 2004). Foliar symptoms of Petri disease and/or esca were not observed in either of the trials during assessment.

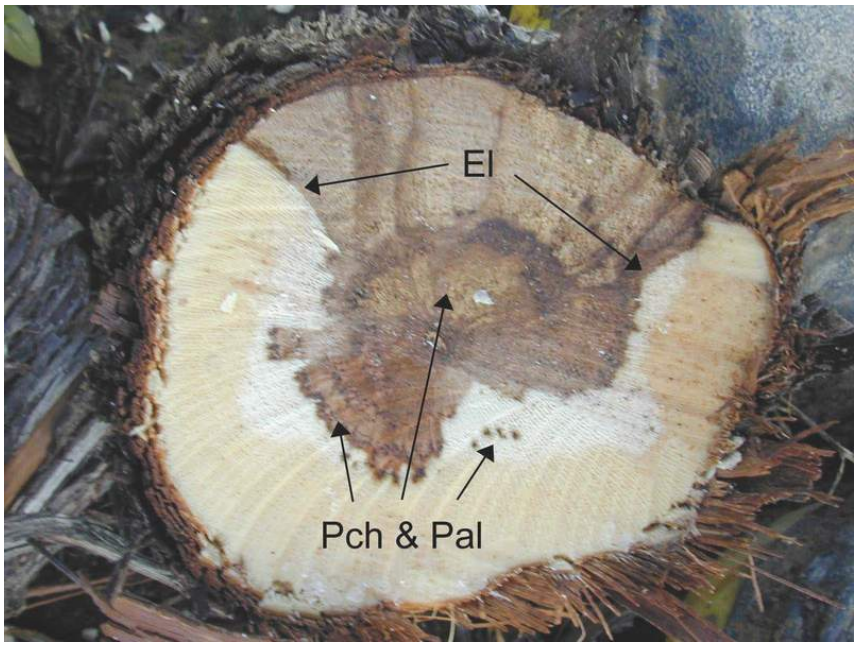


Figure 6.30. Cross–section of a Shiraz grapevine trunk cut 30–40 cm above the ground showing characteristic wood discolouration (Trial 7). Arrows indicate the locations from which the following fungi were isolated; *E. lata* (El), *P. chlamydospora* (Pch) and *P. aleophilum* (Pal).

This research has demonstrated that the short–term effectiveness of remedial surgery for managing eutypa dieback–affected vines is influenced by cultivar and the extent of infection. Pinot Noir and Malbec produced watershoots more readily than Shiraz and Cabernet Sauvignon, when cut 30–40 cm above the ground during remedial surgery, confirming that cultivars differ in their ability to produce watershoots. Furthermore, more vines produced watershoots when cut at the crown than when cut closer to the ground. However, when cut at the crown, 32 to 40% of watershoots originated more than 40 cm above ground level, increasing the likelihood that *E. lata* infection exists beneath the watershoot. Remedial surgery on Shiraz vines in Trial 1 initially appeared unsuccessful, given that 71% of vines contained wood symptoms following surgery and only 65% of vines produced watershoots. However, 6 years later, the incidence of watershoots had increased to 80% and only 5.8% of vines showed recurrence of foliar symptoms. In Trials 2 and 3 at McLaren Vale, recurrence of foliar symptoms was greater in Shiraz than in Malbec vines. The management of both cultivars was the same, so the difference in foliar symptoms may reflect the greater susceptibility of Shiraz than Malbec.

Foliar symptoms were recorded in the first season following surgery in Trial 5, which was observed to have 56% of vines with foliar symptoms before the train and cut method of surgery was used. This suggests that watershoots may have originated above wood infected with *E. lata* and hence were affected by toxins, although no data were obtained on the extent of wood staining in the trunks. Three of four trials which were conducted using the train and cut method had vines which displayed foliar symptoms within 3 years and four of six trials conducted using the cut and train method were symptomless for at least 3 years following surgery. These results indicate that, in the short–term, the cut and train method reduces recurrence of foliar symptoms compared to the train and cut method.

However, the number of vines with recurring foliar symptoms is low, so it will be difficult to draw conclusions about vineyard sustainability until long–term assessments of these trials are completed.

The recurrence of foliar symptoms on reworked vines was not limited to trunks with a wedge–shaped stain remaining in the stump cross–section after remedial surgery. This suggests (i) that the trunk may have had a separate infection below the wound, (ii) that *E. lata* may have colonised the wood in advance of the stained tissue, or (iii) that a new infection had occurred since surgery, possibly through the large trunk wound.

Foliar symptoms were not a reliable indicator of the location or the extent of *E. lata* infection within a vine. For instance in Trial 1, foliar symptoms were recorded in 35% of vines before surgery, however, wedges of stained wood were recorded in 100% of cordons. This concurs with observations that foliar symptoms varied from season to season and infected vines did not always express foliar symptoms (see section 6.1.3).

In Trials 7 and 8, although the causal fungi were isolated from symptomatic wood, it is important to note that the external symptoms of Petri disease (young vine decline, shoot dieback) and esca (tiger striped foliage, apoplexy) have not been observed in these vineyards and are rare in SA. It is possible that severe stress induces foliar symptoms of these diseases once the fungi have blocked the vascular system in the trunk. However, foliar symptoms of eutypa dieback are commonly observed in the two vineyards, suggesting that expression of foliar symptoms is not reliant on severe stress.

6.3.2 Grafting

At least one of the two grafts was successful in 81% of vines and 45% of vines had two successful grafts. Of the vines where both grafts failed, 50% produced watershoots. There was no significant effect of pruning wound treatment on the success of grafting. In 2005, four years after vines were grafted, foliar symptoms were observed in 4.5% of vines. Symptoms were expressed in non–inoculated vines and vines that had been treated with either Nustar or Trichoseal, however, no symptoms were recorded on inoculated controls.

In the short–term, grafting appeared successful, with over 81% of vines retaining at least one successful graft and only 4.5% of vines displaying foliar symptoms after four years. Wound treatments did not affect watershoot production or recurrence of foliar symptoms. As with remedial surgery, long–term assessment will be necessary to draw conclusions on the success of grafting as a control strategy. Grafting provides growers with the opportunity to replace the cultivar to respond to changes in consumer demand for wine, whilst also perhaps controlling eutypa dieback–affected vines.

6.3.3 Injection trials

The experimental chemicals did not influence yield or expression of foliar symptoms relative to the untreated and water–injected controls over the 3 years following treatment. This may be due to poor distribution of chemicals around the trunk, especially into dead tissue where cells can no longer facilitate the movement of compounds in plants. It is also possible that the experimental chemicals were not effective against *E. lata* in the vine and the use of alternative chemicals may have provided some effect.

6.3.4 Brotomax trials

Eutypa dieback was well established in both vineyards. In the first year of the study, 68 and 53% of the vines displayed symptoms of the disease in Eden Valley and McLaren Vale, respectively. In the first 2 years, yield from Brotomax–treated vines was marginally, but not significantly, greater than that of untreated vines (Figure 6.31A). In 2003–04, there was a significant yield increase of 20% for vines treated with Brotomax compared to untreated vines, which reflected increased bunch numbers. However, in 2004–05, the yield difference between treated and untreated vines was not significant.

The severity of foliar symptoms of eutypa dieback was not reduced in any of the 4 years of treatment with Brotomax (Figure 6.31B).

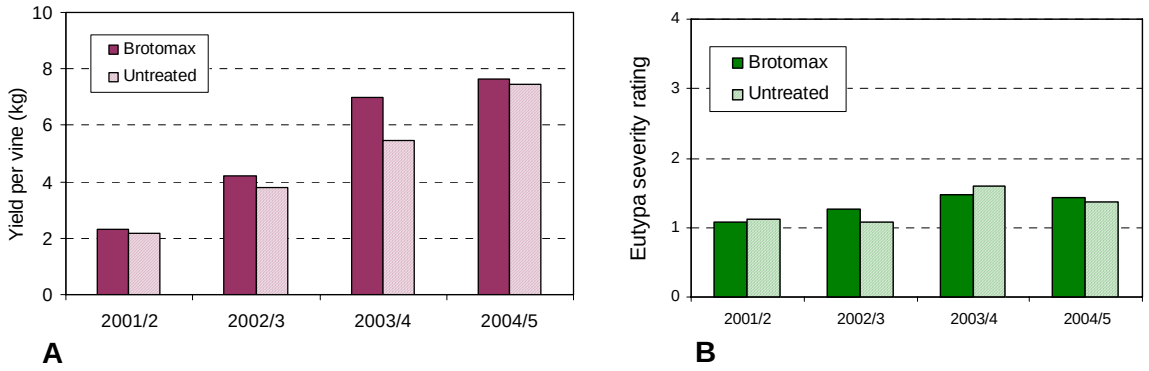


Figure 6.31. The average yield (A) and eutypa dieback foliar symptom severity rating (B) of vines at Eden Valley and McLaren Vale (data combined) from 2001 to 2005. Treated vines were sprayed until runoff with approx 1.5 L of Brotomax (10 mL/L) using a backpack sprayer.

Application of Brotomax to eutypa dieback–affected vines for three seasons led to an average yield increase of 20% over the two sites in the third season, but there was no effect on foliar symptoms. However, in the fourth season there was no difference in yield, which may be due to the mild and wet year, which may alleviate stress on vines and yield loss due to eutypa dieback.

6.3.5 *Trichoderma* trials

Results from each trial over several years of assessment showed that there was no difference in foliar symptoms between vines treated with *Trichoderma* spp. and control vines. Vines from Trial 1 were harvested in 2005 and there was no yield difference between treated and control vines.

Application of *Trichoderma* spp. to eutypa dieback–affected vines, either by injection or inserting impregnated dowels, had no apparent impact on foliar symptoms within 9 years of application. It is important to note that injection of benomyl into the trunk also had no effect on eutypa dieback foliar symptoms, even though it can prevent infection by spores when applied to pruning wounds (section 6.2.1). It may be that both the biocontrol agent and the fungicide were not distributed to all parts of the trunk, especially necrotic tissue colonised by *E. lata*. John *et al*. (2005a) showed that *Trichoderma* colonised up to 18 cm above the dowel 20 months after insertion of trichodowels into healthy vines. It is possible that if younger vines, not infected by *E. lata*, were inoculated, this would enable *Trichoderma* to colonise the trunk, and protect against future infection. Trials using vines with no previous symptoms, such as Trial 5 in Eden Valley along with two trials where Vinevax dowels were inserted into trunks of apparently healthy Pinot Noir and Gamay vines at Nuriootpa Research Centre in 2003, may provide us with this information.

6.4 Diagnosis of eutypa dieback

6.4.1 Development of DNA markers specific to *E. lata*

6.4.1.1 Development of SCAR markers for a PCR–based assay

Both sets of SCAR primers generated PCR products of the expected size using DNA from three isolates of *E. lata*. One primer pair (designated Eut02 F3/Eut02 R2) was selected for further analysis. DNA amplification using this primer pair resulted in fragments of the expected size (approximately 650 bp) from all isolates of *E. lata* tested, but not from any other species (Figure 6.32). This primer pair was, therefore, considered specific to *E. lata* and was selected for use in subsequent PCR analysis.

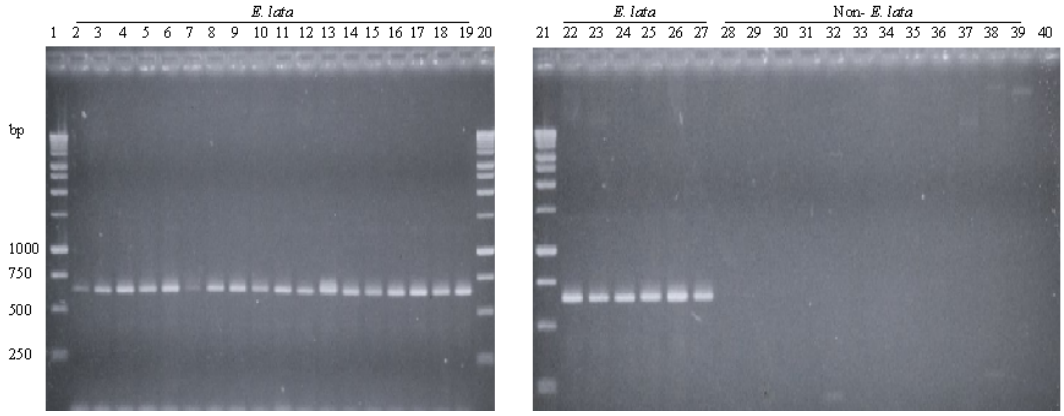


Figure 6.32. PCR amplification of DNA from *E. lata* and non–*E. lata* isolates using SCAR primer pair Eut02 F3/Eut02 R2. Lanes 1, 20, 21 Promega 1 kb DNA ladder; lanes 2–19, M280, M295, M302, N01, N03, N04, N07, N08, AsW, cab2, SS2, SS4, SS6, SS10, SS11, Nuri, HT01, Knox; lanes 22–39, JB16, A166, P#1ss12, EVS, 1776, SS357–3, C2a, C2b, W01, W05, EV01, 1785, 1735, 1739, 1740, 1750, 1765, 1775, grapevine, dd H2O.

6.4.1.2 Assessment of SCAR and ITS markers developed in France and California

Preliminary analysis showed that only one of six primer pairs (SCA 10A and SCA 10B) designed by Lecomte *et al.* (2000) generated PCR products of the expected size (350 bp) from DNA of all *E. lata* isolates tested and not from any non–*E. lata* isolates. However, further studies showed that this primer pair also amplified DNA from non–*E. lata* isolates E178 and SS1#1 and, hence, could not be considered specific to *E. lata*. Similarly, analysis of the primer pair developed at E. & J. Gallo Winery showed that DNA from species other than *E. lata* was also amplified.

The lack of specificity of the French and Californian PCR primers may reflect genetic variation among populations of *E. lata* from different locations. The lack of specificity of the French primer pairs was confirmed by Rolshausen *et al.* (2004) who noted that they also amplified DNA from other Diatrypaceous fungi. The primers developed at E. & J. Gallo Winery, along with three of the French primer pairs, were based on ITS DNA sequence data. A search of the GenBank database by Rolshausen *et al.* (2004) showed that other Diatrypaceous fungi have similar ITS regions, which may explain the lack of specificity of these primer pairs.

Specific SCAR primer pair Eut02 F3/Eut02 R2 was tested against DNA of isolates from Europe, South Africa and the USA, and appeared ubiquitous and specific to *E. lata*. However, further testing using more *E. lata* and non–*E. lata* isolates from other countries would be required to confirm that this is definitely the case. A material transfer agreement was signed, allowing South African researchers to use this primer pair, however, results of their research are not yet available.

6.4.1.3 *Development of markers for a Southern hybridisation–based assay*

Analysis of the genomic DNA library showed that one clone was specific to *E. lata* and gave a strong signal towards DNA from pure cultures of the pathogen following 2–3 days of exposure to X–rays at –70°C. This clone (designated clone C68) was selected for use as an *E. lata*–specific DNA probe. The remaining clones were either not specific, or gave an extremely weak signal following prolonged X–ray exposure at –70°C.

6.4.2 Detection of *E. lata* in grapevine wood using DNA markers

6.4.2.1 *PCR–based detection of* E. lata

Only the Bio-101 FastDNA SPIN kit for soil enabled reliable amplification of DNA from grapevine wood known to be infected with *E. lata*. The remaining protocols did not permit the consistent amplification of *E. lata* DNA from wood known to be infected with the pathogen, even after seeding these extracts with DNA from pure cultures of *E. lata*. Addition of PVP, a compound known to enhance the efficiency of the reaction, facilitated the generation of amplification products, but not consistently. Similarly, fluorescence–based PCR analysis of DNA extracted using automated procedures at the AGRF showed that none of the procedures yielded DNA of sufficient quality for use in such analysis.

It is likely that phenolic compounds which inhibited the PCR were present in DNA extracts obtained using protocols other than the Bio-101 extraction kit. The Bio-101 soil DNA extraction kit costs approximately $20 per DNA extraction, which, when added to the labour cost and the cost of the PCR itself, meant that it was not a cost effective technique for identifying *E. lata* in the present study. Because a PCR–based assay remains desirable, given its potential to be more rapid and sensitive than the slot blot assay (see below), it was hoped that the automated DNA isolation protocols would provide DNA suitable for use in high–throughput fluorescence–based PCR analysis. However, DNA of suitable quality could not be obtained, presumably because of contamination by phenolic compounds. It remains possible that, as the technology associated with automated DNA analysis improves, suitable methods may become available in the future.

6.4.2.2 Southern hybridisation–based detection of E. lata

Validation of the slot blot assay using DNA isolated from inoculated canes enabled the detection of *E. lata* in nine of 10 inoculated samples but not in uninoculated controls (Figure 6.33). Mycelium of *E. lata* was cultured from only two of the 10 inoculated canes. Slot blot analysis of DNA isolated from 11 symptomatic, naturally infected grapevine trunks detected *E. lata* in each case.

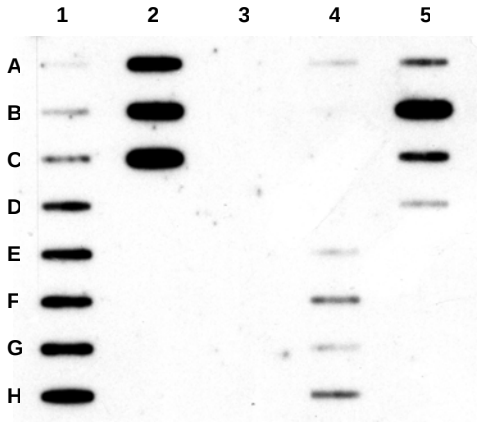


Figure 6.33 Detection of *E. lata* in DNA extracted from inoculated canes by slot blot analysis using the *E. lata*–specific clone, C68. Slots 1A–2C contain increasing concentrations of genomic DNA from *E. lata* isolate M302; 1, 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, 50 ng respectively. Slots 2D–2H contain dd H_2O. Slots 3A–3F contain genomic DNA from grapevine, non–*E. lata* isolates 1739, 1740, 1750, 1775, 1785. Slots 3G and 3H contain dd H_2O. Slots 4A and 4B, 4E–5D contain 200 ng total DNA obtained from inoculated grapevine canes. Slots 4C and 4D contain 200 ng total DNA obtained from uninoculated grapevine canes.

Comprehensive validation of the probe using samples from 476 vines (section 6.3.1) showed that the DNA probe was consistently more sensitive than identification based on culturing mycelium from wood (Table 6.6). Analysis of 1250 samples from pruning wound trials showed that in some treatments the DNA probe detected *E. lata* in a greater percentage of canes than isolation, but in other treatments the converse was the case (Figure 6.34).

Table 6.6 Comparison of DNA–based and isolation data for detecting *E. lata* in mature wood

Trial site	Percentage of vines from which *E. lata* was recovered	
	DNA	Culture
Eden Valley	51	21
McLaren Vale	54	39
Coonawarra	64	10

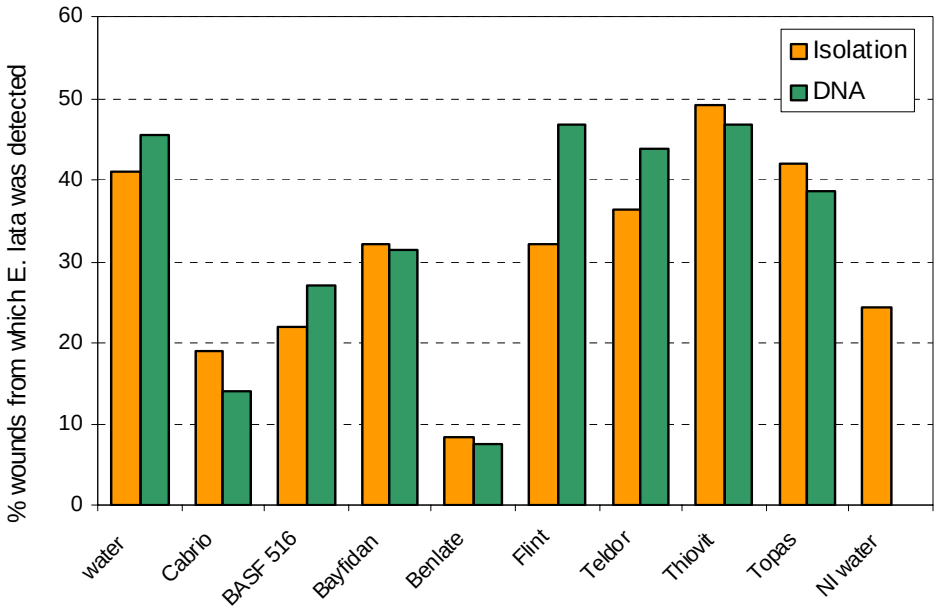


Figure 6.34 Comparison of recovery of *E. lata* from annual canes using isolation of mycelium or the DNA probe. Canes were inoculated with *E. lata* one day after treatment of wounds by painting and spraying. NI water = not inoculated (water control).

Analysis of the 476 samples from mature vines confirmed that the DNA probe was considerably more sensitive and reliable than identification based on culturing woodchips, illustrating the potential of the *E. lata*–specific probe for use in epidemiological studies. However, slot blot analysis of 1250 samples from the pruning wound trial was more ambiguous, with culturing appearing more sensitive than the probe in some instances. However, the identity of cultures isolated from wood was based on morphology of mycelium only. When the pruning wound trial was established, ascospores of a fungus indistinguishable from *E. lata* may have been isolated from some of the infected wood used as the source of inoculum in these trials, and subsequently used as inoculum for pruning wounds. That fungus may then have been isolated from the canes when the trial was assessed, and misidentified as *E. lata*. The occurrence of *E. lata*–like ascospore–producing fungi on grapevine wood, and the similarity of ascospores and mycelium of this unidentified species to those of *E. lata*, highlight the value of using the specific DNA markers to confirm the identity of cultures as *E. lata*. Based on these results, it is considered that the DNA probe provides accurate information on the presence of *E. lata* in infected wood, and should be used to assess such trials in the future.

6.4.3 Identification of *E. lata* from mycelium using SCAR markers

PCR products of the expected size (650 bp) were obtained from each *E. lata* isolate but no others when DNA was amplified from mycelium using the protocol of Hamelin *et al.* (2000) (Figure 6.35). Analysis of 80 putative *E. lata* isolates obtained from vines in McLaren Vale, and identification by morphology, showed that 76 of 80 isolates were, in fact, *E. lata*.

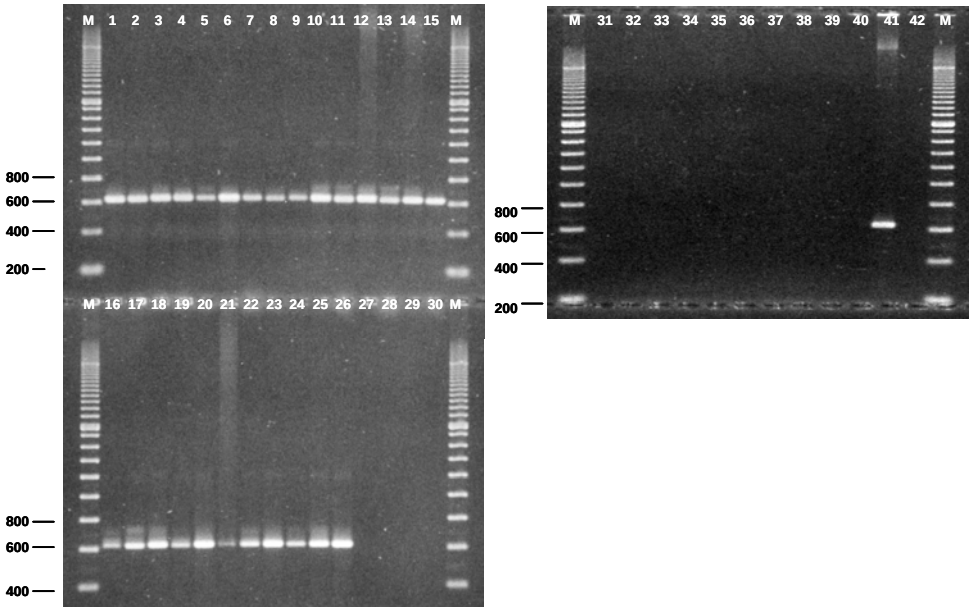


Figure 6.35 PCR amplification of *E. lata* DNA isolated from mycelium using the protocol of Hamelin *et al.* (2000), with SCAR primers specific to *E. lata*. Lanes 1–26, mycelial extract from 26 isolates of *E. lata*; lanes 27–40, mycelial extracts from 14 non–*E. lata* isolates; lane 41, genomic DNA from *E. lata* isolate M280; lane 42, PCR negative control. M, molecular weight marker (200 bp ladder, Geneworks Pty Ltd), size in bp indicated on the left.

The suspected mis-identification of other species as *E. lata* in the pruning wound trial highlights the benefits of using the SCAR markers to validate the identity of cultures suspected to be *E. lata*. Indeed, the SCAR markers were routinely used in the project since 2003 to confirm the identity of ascospore–derived isolates used to inoculate vines in pruning wound trials and to identify isolates obtained from diseased samples. This technique is more reliable than identification based on morphology and is also much quicker, in that *E. lata* can be identified from 5–7 day old cultures on agar. Identification of mycelial cultures based on morphology may take 2–3 months.

The observation that four of 80 isolates obtained from cankers in mature vines and identified as *E. lata* based on morphology were not, in fact, *E. lata*, suggests that other fungi may also induce internal symptoms of eutypa dieback in Australia. The related species, *Eutypa leptoplaca*, has been isolated from necrotic tissue on grapevines in California and is pathogenic in the glasshouse (Trouillas and Gubler, 2004). Further research should be conducted to establish the existence of this pathogen in Australia and, if found, its role in causing disease. Results of this research were published in Mycological Research (Lardner *et al.*, 2005). A copy of this manuscript is included in Appendix 1.

6.4.4 Genetic *variation* in *E. lata*

6.4.4.1 RAPD analysis

RAPD analysis of 11 isolates received as *E. lata* generated 107 scoreable bands and identified 11 haplotypes. Neighbour joining analysis indicated that isolates E178 and SS1#1 were significantly different both from each other and from all other isolates. The remaining nine isolates were placed in a single group, which was strongly supported by bootstrap analysis.

6.4.4.2 RFLP analysis

Thirty–five RFLP phenotypes were resolved among the 38 isolates. Three isolates from New Zealand (SS1#1, SS1#3 and SS1#9) shared identical phenotypes. Neighbour joining analysis separated these isolates from all others, suggesting that they were not *E. lata*. The remaining isolates, with the exception of E178, were placed in a single group which was strongly supported by bootstrap analysis (Figure 6.36). There was no correlation between host or geographic origin of isolates and genetic profiles.

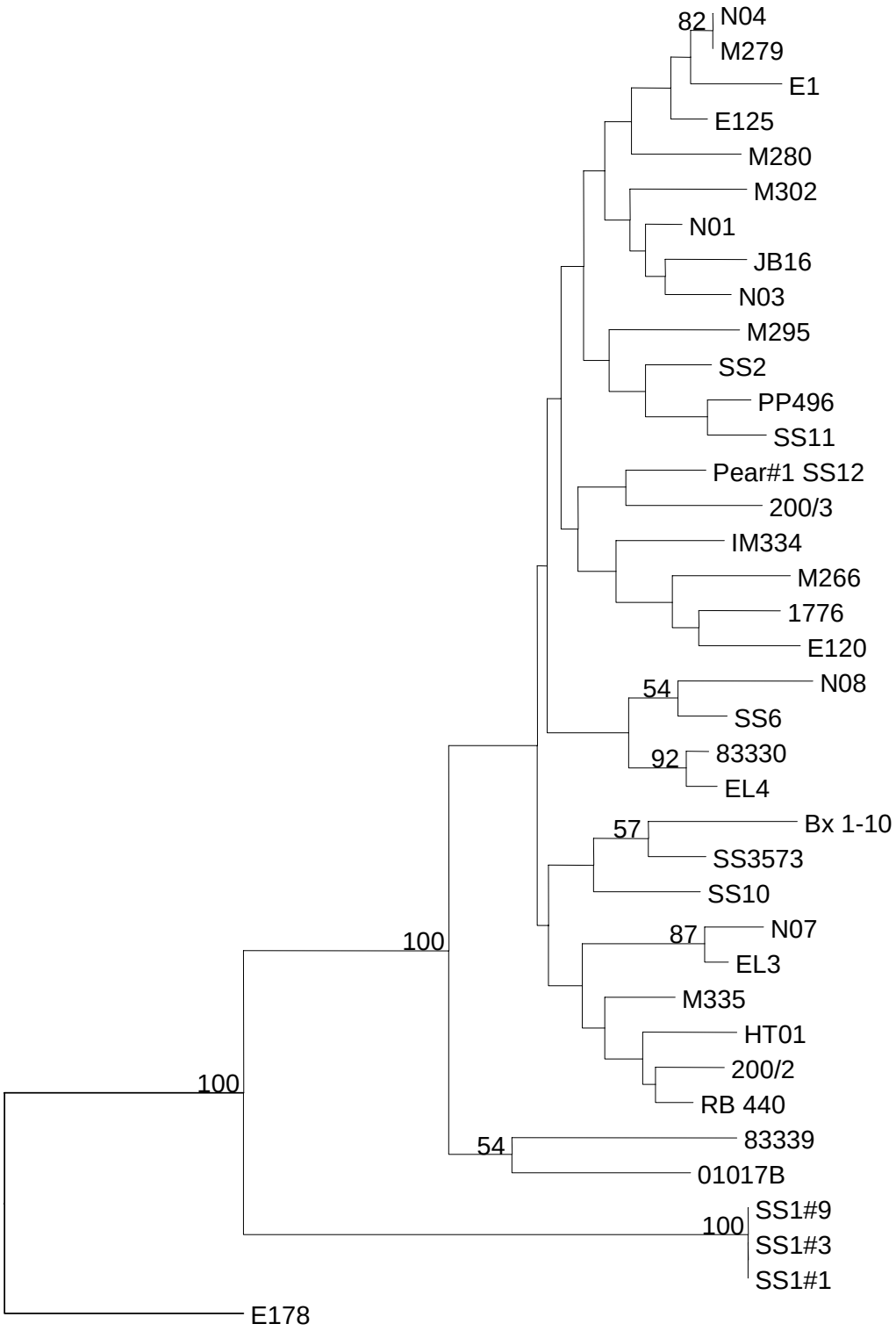


Figure 6.36 Neighbour joining tree generated using TREECON (Van de Peer and De Wachter, 1994), showing RFLP data from 38 isolates following digestion with restriction enzymes *Eco* RI and *Pst* I and labelling with six probes. Isolate E178 was specified as the outgroup. Distances were calculated using the algorithm of Nei and Li (1979). Bootstrap values greater than 50% are given.

Comparison of neighbour joining analysis of molecular and biochemical data (section 6.4.5.2) showed no correlation between secondary metabolite production and genetic variation in *E. lata*. Furthermore, there was no apparent relationship between host or geographic origin of isolates of *E. lata* and the amount or type of secondary metabolite produced.

The lack of correlation between molecular profiles and geographic origin or host species supports Péros *et al.* (1996) and DeScenzo *et al.* (1999), who observed no correlation between geographic

origin and RAPD banding patterns or AFLP and rDNA ITS sequence data, respectively. The high degree of genetic diversity seen here is not surprising, given that *E. lata* is primarily dispersed by ascospores and that perithecia are believed to result from outcrossing (Péros and Berger, 1999, Cortesi and Milgroom, 2001).

This is the first study to compare secondary metabolite production and genetic profiles of *E. lata*. There was no apparent correlation between these factors in the current study. Similar studies of other fungal species have found a correlation in some cases (Stadler *et al.*, 2001, Mule *et al.*, 1997), but not in others (Tran–Dinh *et al.*, 1999, Egel *et al.*, 1994). The lack of correlation between genetic profiles and secondary metabolite production observed here may be due to the relatively small number of DNA markers used, and the random nature of these markers.

6.4.5 Production of phenolic secondary metabolites by *E. lata*

6.4.5.1 Identification of culture media and preliminary assessment of secondary metabolite production by E. lata

Secondary metabolite production on artificial media

Forty one different phenolic secondary metabolites were detected following growth of 11 isolates of *E. lata* on liquid and solid artificial media. These included eutypinol, siccayne, eulatachromene, eutypine, eulatinol and 2-iso-propenyl-5-formylbenzofuran, hereafter referred to as benzofuran. Each of these compounds has previously been identified in culture filtrates of *E. lata* (Renaud *et al.*, 1989, Molyneux *et al.*, 2002). The remaining 35 compounds were identified by retention times (Rt) only. Nineteen of these 35 compounds were produced by a single isolate only, although not by the same isolate in each case. The structure of these 35 compounds was not elucidated because they were generally produced in low levels and not by all isolates and, hence, were not considered suitable for use as diagnostic markers.

Both medium and environment (shaken or stationary) influenced the amount and composition of secondary metabolites produced by the 11 isolates. However, there was no consistent correlation between these factors and the metabolites produced. No single compound was produced by all isolates, although one compound (Rt 15.1 minutes) was produced by all isolates except M335. However, this compound was present following growth of isolates on malt yeast broth only and, in some instances, was present in trace amounts. Eutypinol was produced by seven of the 11 isolates (E1, E125, M266, M279, M280, N04, and SS6, all confirmed as *E. lata*). Of the other compounds which were characterised for *E. lata*, eutypine was produced by five isolates (E1, E125, M266, M279 and SS6), siccayne by seven isolates (E1, E120, E125, M266, M280, N04 and SS1#1), eulatinol by seven isolates (E1, E120, E125, M279, M280, N04 and SS6), benzofuran by two isolates (E1 and M266), and eulatachromene by one isolate (E125). No other compounds were produced by more than six of the 11 isolates.

Secondary metabolite production on grapevine extracts

HPLC analysis of the 11 isolates grown on grapevine cane extract identified 29 phenolic secondary metabolites, including eutypinol, siccayne, eulatachromene, eulatinol and eutypine. The remaining 24 compounds were identified by retention time only. No single compound was produced by all isolates, although eutypinol and eutypine were produced by eight isolates (all except E178, M335 and SS1#1). A previously unidentified compound with Rt 19.6 was produced by the same eight isolates. Another unidentified compound (Rt 14.0) was produced by seven isolates (E1, E120, E125, M266, M280, N04 and SS6) and one compound (Rt 16.0) was produced by six isolates (E120, E125, M266, M279, M280 and SS6). Seventeen compounds were produced on a single medium only, and nine of these were produced by a single isolate only, although not by the same isolate in each case. The addition of sucrose to the culture medium increased the amount and variety of secondary metabolites produced.

This preliminary analysis was more comprehensive than previous studies where secondary metabolite production by one to three isolates of *E. lata* was analysed after growth on one or two media (Renaud

et al., 1989, Molyneux *et al.*, 2002). Although 41 different metabolites were detected, most of these were produced by one or two isolates only and, hence, were not considered suitable for use as a diagnostic marker. Production of secondary metabolites increased and was more consistent following growth on milled grapevine cane, as opposed to artificial media, in particular when the cane was amended with sucrose. Consequently, this medium was used in further experiments to identify a chemical marker specific and ubiquitous to *E. lata*.

6.4.5.2 Identification and characterisation of a specific chemical marker

Identification of metabolites specific and ubiquitous to E. lata

Analysis of secondary metabolites produced by 30 isolates of *E. lata* grown on grapevine cane amended with sucrose identified significant quantities of five phenolic metabolites, namely eutypinol, methyl eutypinol, eulatachromene, eutypine and benzofuran. A quinol compound, eulatinol, was also produced by some isolates. Metabolite production by each isolate on each cultivar is summarised in Table 6.7. One isolate (M335) did not produce any metabolites in this, or any other experiment. This isolate may have lost the ability to produce these compounds, perhaps following storage in water, as has been observed in other fungal species (Santos *et al.*, 2002). It is believed that the response of fungi to storage may be isolate–specific (Santos *et al.*, 2002, Ryan *et al.*, 2001), which would explain why this was the only isolate that did not produce any secondary metabolites.

Of the 29 isolates which did produce secondary metabolites, each produced eutypinol, but not necessarily on wood of every cultivar. There was no apparent correlation between cultivar and the amount or type of secondary metabolite produced. On each cultivar, eutypinol was predominant followed by methyl eutypinol and eulatachromene. The total amount of metabolites produced by the 30 isolates was considerably greater on Shiraz and Semillon (9457 and 8379 µg, respectively) than on Merlot (4184 µg). Amounts of eutypinol varied depending on fungal isolate and cultivar. For example, isolate M266 produced 1070, 137 and 13 µg of eutypinol following growth on Shiraz, Semillon and Merlot, respectively. Similar patterns were apparent for other metabolites. Eutypinol comprised 82% of the total metabolites produced when isolates were grown on Shiraz, 88% on Semillon and 90% on Merlot. Methyl eutypinol was also relatively abundant, produced by 25 isolates in amounts ranging from 0.3 µg (SS6, Shiraz) to 259 µg (N07, Shiraz). Taken together, eutypinol and methyl eutypinol comprised approximately 94% of the total acetylenic phenol metabolites produced on each cultivar. Eutypine, previously implicated as the metabolite responsible for foliar symptoms of eutypa dieback, was produced in smaller amounts by 23 isolates. HPLC analysis of culture filtrates from non–*E. lata* isolates showed that these species produced no metabolites in common with *E. lata*, although some produced unrelated compounds.

Although eutypinol is not phytotoxic when applied to grapevine leaf discs (Smith *et al.*, 2003, Mahoney *et al.*, 2003), this does not preclude its use as a chemical marker. This is because pathogenicity of *E. lata* is not solely a consequence of the production of phenolic metabolites, but also reflects the ability of the pathogen to colonise grapevine wood and degrade the xylem tissue, ultimately killing the vine or a significant portion of it. Hence, the presence of *E. lata* in vines as illustrated by a non–toxic metabolite is sufficient indication of infection.

Not every isolate produced eutypinol on every cultivar, and similar patterns of production were seen for other compounds. This is consistent with studies showing that production of certain metabolites by *Fusarium* spp. appears to be species–, isolate– and substrate–specific (Doohan *et al.*, 2003). Factors that influence fungal secondary metabolite production remain unclear (Kokkonen *et al.*, 2005, Betina, 1989), with different compounds commonly produced on different substrates. Indeed, for *E. lata*, eulatinol was produced almost exclusively on artificial media, in particular on MYB, whereas other compounds, for example eutypine and eulatachromene, were more abundant when cultured on grapevine extracts (Mahoney *et al.*, 2003).

Table 6.7 Summary of secondary metabolite production by 30 isolates of *E. lata* on milled cane from three grapevine cultivars

Isolate	Metabolites (µg)														
	Eutypinol			Methyl eutypinol			Eulatachromene			Eutypine			Benzofuran[1]		
	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon
01017B	63	19	13	3.3	1	1.2	—	—	—	1.1	—	—	—	0.4	0.1
1776	2.5	—	1.5	—	—	—	—	—	—	—	—	—	—	—	—
200/2	351	440	586	23	5.2	13	26	28	21	2.9	2.4	—	3	—	0.9
200/3	466	111	615	43	9.4	31	25	—	23	16	—	19	1	6.1	1.1
83330	1.1	1.8	96	—	—	8.8	—	—	10	—	—	2.6	—	—	1.1
83339	661	236	1380	126	7.4	138	28	5.5	36	—	—	—	—	0.5	—
Bx 1-10	197	35	282	59	2	24	—	—	4.9	—	—	5.1	3.5	2.4	1.5
E1	295	56	81	47	5.2	3.8	—	—	—	12	—	0.7	10	0.7	0.6
E120	172	88	180	—	—	—	5.3	5.3	8.8	4.2	0.7	13	0.3	1.9	3
E125	869	255	547	47	1.7	14	24	11	17	57	1.8	—	75	1.9	16
EL3	387	266	130	53	11	15	28	11	3.8	—	1.5	—	1	0.6	0.4
EL4	114	279	103	28	10	4.9	12	21	5.6	27	9.1	1.1	1.2	3.1	0.9
HT01	491	105	417	59	4.8	15	20	1.5	9.3	7.6	2	4.1	2.1	1.4	1.3
JB16	706	486	701	164	43	84	33	16	47	—	19	—	—	12	—
M266	1070	13	137	—	—	1.3	—	—	1.3	18	—	—	—	0.1	1.9
M279	—	7.8	20	7.0	6.2	27	—	1	—	—	—	7.3	—	2.7	27
M280	179	522	491	11	8.5	—	0.8	6.9	7.3	2.7	4	18	12	11	—
M295	—	—	1.4	—	—	—	—	—	—	—	—	—	—	—	—
M302	131	41	75	9.6	1.5	2.6	4.2	—	1.8	12	—	1.2	1.2	0.4	0.1
M335	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
NO3	303	19	82	51	0.9	3.9	—	—	—	—	—	—	10	0.4	4.2
NO4	610	199	487	96	21	55	13	4.5	11	18	—	—	6	3.6	12
NO7	216	224	310	259	36	85	9.7	8.7	7.8	—	—	—	10	0.3	1
NO8	421	39	376	23	1.6	9.2	12	—	8	9.4	—	—	2.1	0.5	9
P#1 SS12	—	25	19	—	—	—	—	—	—	—	—	—	—	0.4	0.1
RB440	24	203	126	7.2	18	21	—	4.7	3.5	7.2	4.2	12	2	2.2	3.6
SS2	—	—	48	—	—	4.5	—	—	1.6	—	10	1.3	—	—	37
SS6	3.8	16	18	6.1	2.3	7.3	—	—	—	1.4	1	3.1	2.7	6.3	9
SS10	3.8	44	17	0.3	2	1	—	—	—	0.05	—	—	—	0.3	0.1
SS11	2	12	9.3	0.5	1.3	1.2	—	—	—	—	0.2	0.3	0.2	—	—
	7738	3745	7350	1124	199	572	241	125	229	196	56	89	144	59	132

— = not detected
1 = 2-*iso*-propenyl-5-formylbenzofuran
Detection limits (ug/ml) at 254 nm: Eutypinol 0.03 Methyl eutypinol 0.03 Eulatachromene 0.05 Eutypine 0.01
Benzofuran 0.005 Eulatinol 0.01 Siccayne 0.01

The pathogen was grown on wood of three cultivars to see if this influenced secondary metabolite production. Although secondary metabolite production varied among cultivars, there was no apparent correlation between the amount or type of metabolite produced and cultivar susceptibility to eutypa dieback. Growing the pathogen on milled cane is expected to reflect secondary metabolite production *in planta*. However, this is still an artificial situation and other factors may influence metabolite production in living vine tissue and, hence, help determine cultivar susceptibility to eutypa dieback. For example, enzymatic detoxification of eutypine to eutypinol in living grapevine tissue, as reported by Colrat *et al.* (1999), would not occur on autoclaved cane. Likewise, the age of the cane may influence the metabolism of the pathogen; when growing *E. lata* on annual cane and mature wood, Ferreira (1999) and Schmidt *et al.* (1999) observed differences in growth rates and production of extracellular hydrolytic enzymes, respectively. Hence, it seems likely that the age of the wood on which *E. lata* is grown would influence production of acetylenic phenol metabolites.

Phytotoxicity of secondary metabolites and correlation with pathogenicity in the shadehouse

Following growth on stationary PDB, filtrates from *E. lata* isolates E1, E120, E125 and M335 and non–*E. lata* isolate E178 showed marked toxicity towards the leaf discs. Filtrates from all isolates grown in shaken PDB were toxic towards leaf discs, even though not all isolates produced detectable quantities of acetylenic phenol secondary metabolites. Similar results were obtained for filtrates of cultures grown in all other media. In no instance did control discs exhibit phytotoxicity. Discs treated with a mixture of four characterised secondary metabolites from *E. lata* (positive control) developed a clearly defined area of necrosis in the centre.

Extracts from six isolates (E1, E125, M266, M280, M335 and N04) were toxic to leaf discs following growth on ground grapevine cane without sucrose. The extract from uninoculated grapevine cane showed no toxicity towards leaf discs. However, the methanol–treated control disc (negative control) and the disc treated with the mixture of four characterised secondary metabolites from *E. lata* (positive control) developed a clearly defined area of necrosis. Following growth on the same medium amended with sucrose, extracts from all 11 isolates exhibited marked toxicity towards leaf discs, although the grapevine extract and methanol controls did not, whereas discs treated with the mixture of four standard compounds developed a clearly defined area of necrosis. It is noteworthy that the two non–*E. lata* isolates were indistinguishable from *E. lata* in this bioassay, implying that these non–*E. lata* isolates, which were also isolated from grapevine wood, may have the potential to be pathogenic to grapevine.

There was no apparent correlation between the amount or type of secondary metabolites produced in culture and pathogenicity in the shadehouse (Table 6.8 and section 6.1.4). For example, isolate 1776, which induced symptoms on 26% of vines, produced a total of 1.3 µg of eutypinol when grown on wood of the three cultivars in artificial culture, whereas isolate N04, which induced symptoms on only 4% of vines, produced a total of 511 µg of five different metabolites; similar discrepancies between the amount of metabolites produced and pathogenicity were observed for other isolates examined here (Table 6.8 and section 6.1.4).

The observation that extracts from cultures of *E. lata* and the two unidentified fungi were toxic to grapevine leaf discs, even when no secondary metabolites were detected by HPLC, implies that compounds other than acetylenic phenols may contribute to the foliar symptoms of eutypa dieback. This hypothesis is supported by the lack of correlation between expression of foliar symptoms *in planta* and secondary metabolite production *in vitro*. Recent research (Octave *et al.*, 2006) showed that a single isolate of *E. lata* produced phytotoxic polypeptides when grown in artificial culture. This provides further evidence to support the observation that other compounds are, at least partially, responsible for the foliar symptoms of the disease. However, additional research would be required to confirm the role of such compounds in disease expression.

Table 6.8 Relationship between pathogenicity in the shadehouse and secondary metabolite production in artificial culture of 12 isolates of *E. lata*.

Isolate	% of plants displaying symptoms	Average metabolite production (μg for 3 cultivars)					
		total	eutypinol	eulatachromene	methyleutypinol	eutypine	benzofuran
1776	26	1.3	1.3	0.0	0.0	0.0	0.0
200/3	4	455.0	397.2	16.0	27.5	11.5	2.7
83330	0	40.3	32.8	3.4	2.9	0.9	0.4
E1	0	170.7	144.1	0.0	18.6	4.2	3.8
M279	0	35.3	9.3	0.3	13.4	2.4	9.9
M280	0	424.7	397.3	5.0	6.5	8.2	7.7
M295	0	0.5	0.5	0.0	0.0	0.0	0.0
M302	26	94.0	82.4	2.0	4.6	4.5	0.6
M335	0	0.0	0.0	0.0	0.0	0.0	0.0
N04	4	511.9	432.0	9.4	57.3	6.1	7.1
SS6	4	25.7	12.6	0.0	5.2	1.8	6.0
SS10	33	22.9	21.7	0.0	1.1	0.0	0.1

Rapid detection of E. lata *with spectroscopy and chemometrics*

HPLC analysis of the 20 isolates selected for spectroscopy and chemometric analysis showed that only 12 produced detectable quantities of secondary metabolites following growth on MYB. Eutypinol was predominant, produced by 11 isolates (Table 6.9).

Table 6.9 Summary of secondary metabolite production by 20 isolates of *E. lata* grown on MYB for 20 days as detected using HPLC.

Isolate	Metabolites (μg/mL)					
	Eutypinol	Eulatachromene	Methyl eutypinol	Eutypine	Eulatinol	Benzofuran
EL3	155.0	9.0	18	0	0	0
E120	12	0	0	0	0	0
E125	223.5	15.5	11.5	0	0	0
M266	386.0	7.0	0	4.0	0	0
CC260	0	0	0	0	0	0
RB440	116.5	5.0	27	0	3.0	0
Bx1-10	64.5	0	5.5	0	0	0
200/3	334.5	24.5	0	40.0	0	13.5
M280	144	3.5	0	1.0	0	2.5
1776	0	0	0	0	0	0
M335	0	0	0	0	0	0
200/2	63.5	9.0	4.5	0	2.5	0
P#1SS12	0	0	0	0	0	0
01017B	0	0	0	0	0	0
N01	0	0	0	0	0	0
SS2	0	0	0	0	0	0
SS6	0	0	4.5	0	0	10.5
HT01	13.0	0	3.0	0	0	3.5
83339	427.0	0	56.0	0	0	0
83330	0	0	0	0	0	0
Control	0	0	0	0	0	0

Spectroscopic analysis showed that the spectra of the high absorbance samples had strong eutypinol features, notably maxima at 262, 276 and 308 nm (Figure 6.37). The overall UV absorbance of samples varied distinctly, and the intensity of peaks observed using UV–Vis spectroscopy correlated with the amount of eutypinol produced by each isolate as detected by HPLC. The un–inoculated control showed higher general absorbance than some inoculated samples, but produced no eutypinol peaks, implying that degradation of UV absorbing material of the culture medium occurred as a result of fungal metabolism. UV–Vis analysis differentiated between the un–inoculated control and inoculated samples which produced no metabolites as detected by HPLC (Table 6.9). These samples could not be differentiated using HPLC; this may have implications for detecting metabolites *in planta* even though metabolites were not detected in infected plant material using HPLC (see section 6.4.5.3).

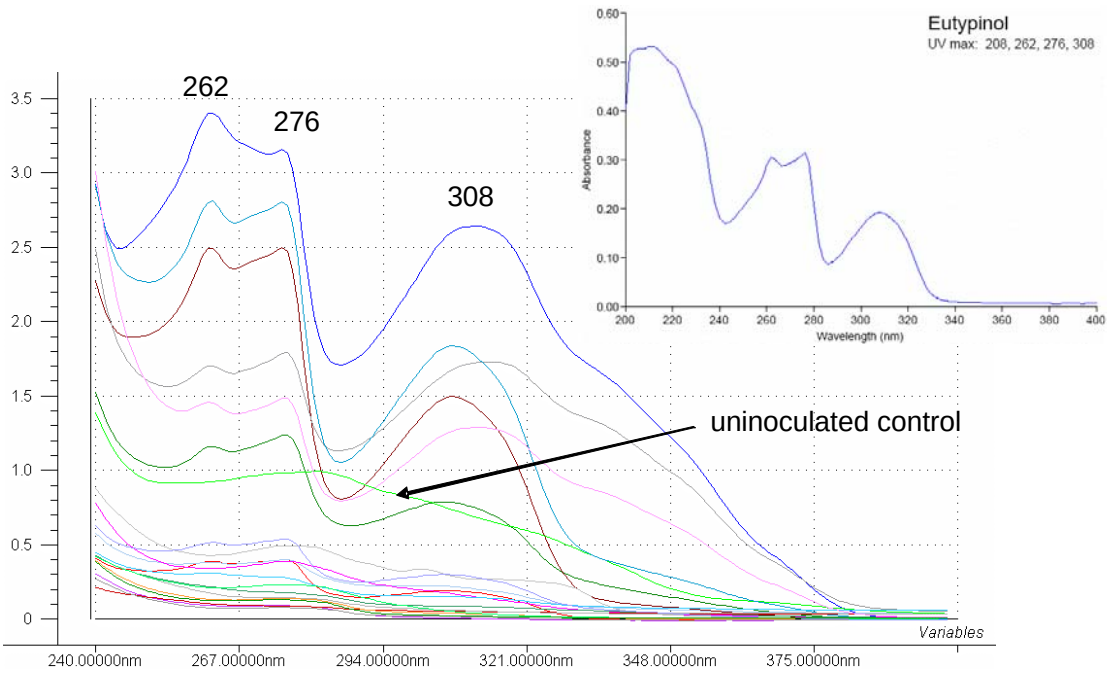


Figure 6.37 UV spectra (240–400 nm) of extracts of un–inoculated control and *E. lata* cultures. The spectrum of eutypinol (200–400 nm) is shown inset

With second derivative treatment of the spectra, baseline shifts are removed and the peaks, although inverted, become better resolved (Figure 6.38). Again, strong eutypinol features can be observed and with the baseline correction the relative heights of the 261 and 276 nm peaks match eutypinol. The control and the sample with the greatest concentration of eutypinol (isolate 83339) are shown in Figure 6.39; with the baseline shifts removed it is clear that the control has no peaks at the eutypinol signature wavelengths.

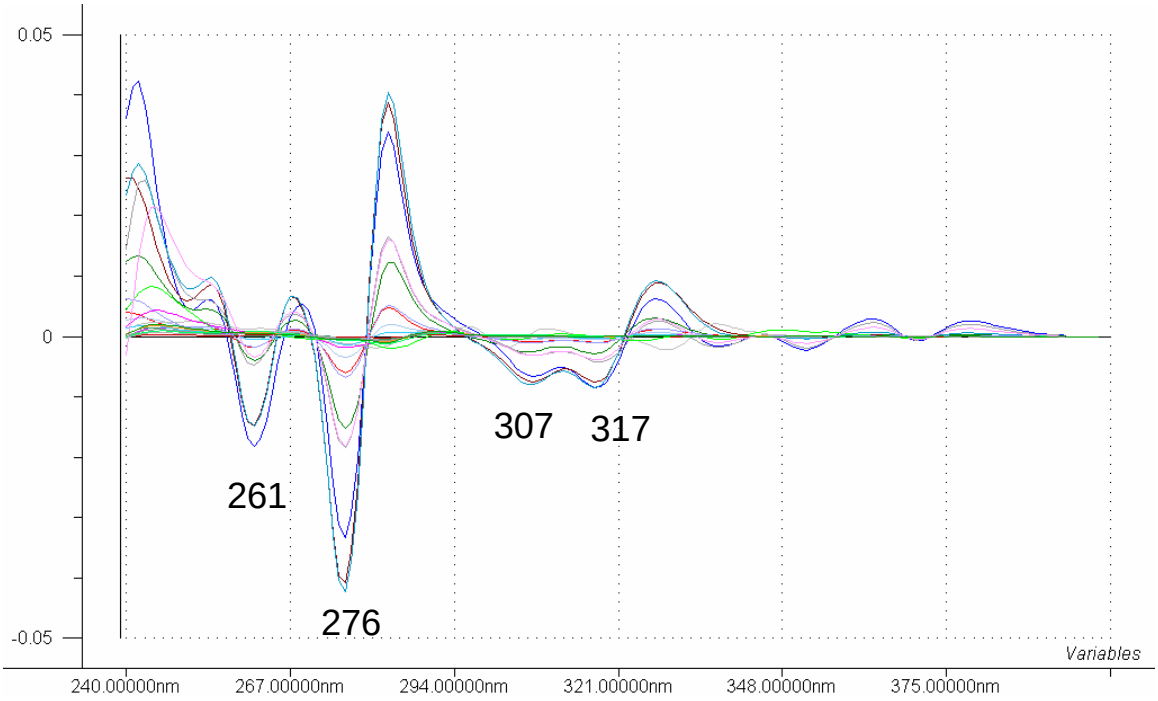


Figure 6.38 Second derivative transformed UV spectra (240–400 nm) of extracts of un–inoculated control and *E. lata* cultures. The major peaks (inverted with 2nd derivative) associated with inoculated media are marked.

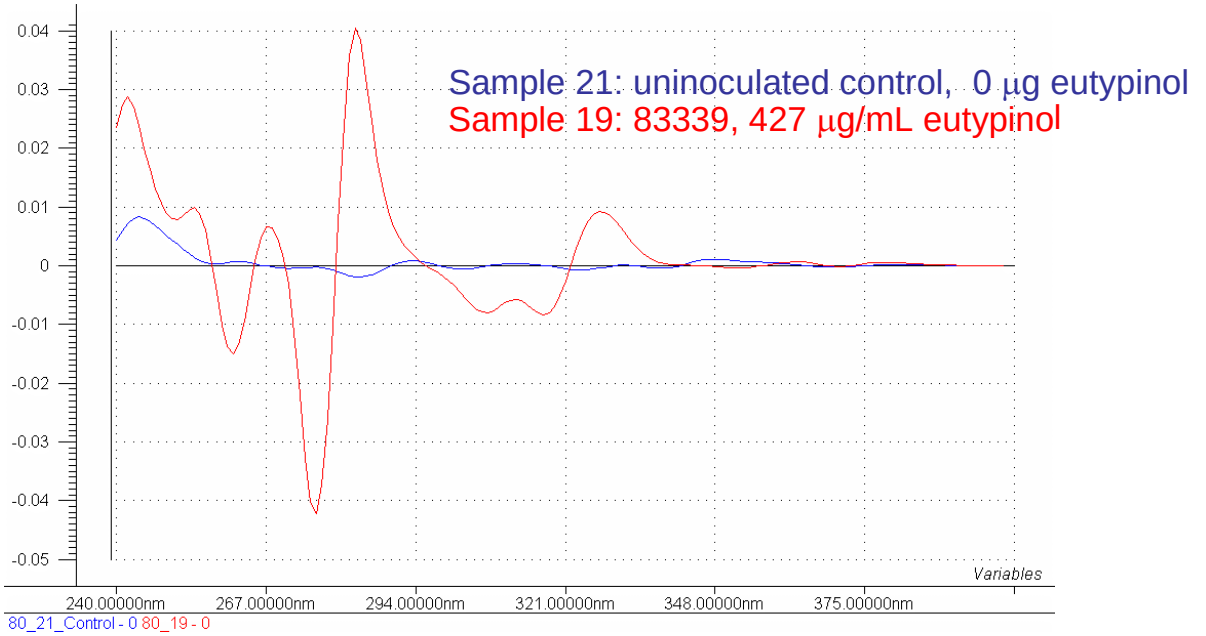


Figure 6.39 Second derivative transformed UV spectra (240–400 nm) of extracts from the un–inoculated control (blue trace, no eutypinol detected) and *E. lata* isolate 83339 (red trace, 427 µg/mL eutypinol).

There were samples that had very high leverage with regard to eutypinol concentration so, rather than prepare a calibration for eutypinol concentration as such, a classification model was examined; this is in tune with the concept of the procedure being used as a disease classification tool. Principal component analysis (PCA) was used to reduce the second derivative spectra and only 3 PCs were required to describe >99.9% of the between–sample spectral differences.

A plot of the first 3 PCs (Figure 6.40) shows that samples are clustered according to the eutypinol concentration. The PCA wavelength loadings are shown in Figure 6.41. Positive and negative loadings match spectra of relevant compounds and the strongest positive loading for the first factor (that explains 93% of the spectral variance) is exactly on a eutypinol peak (276 nm).

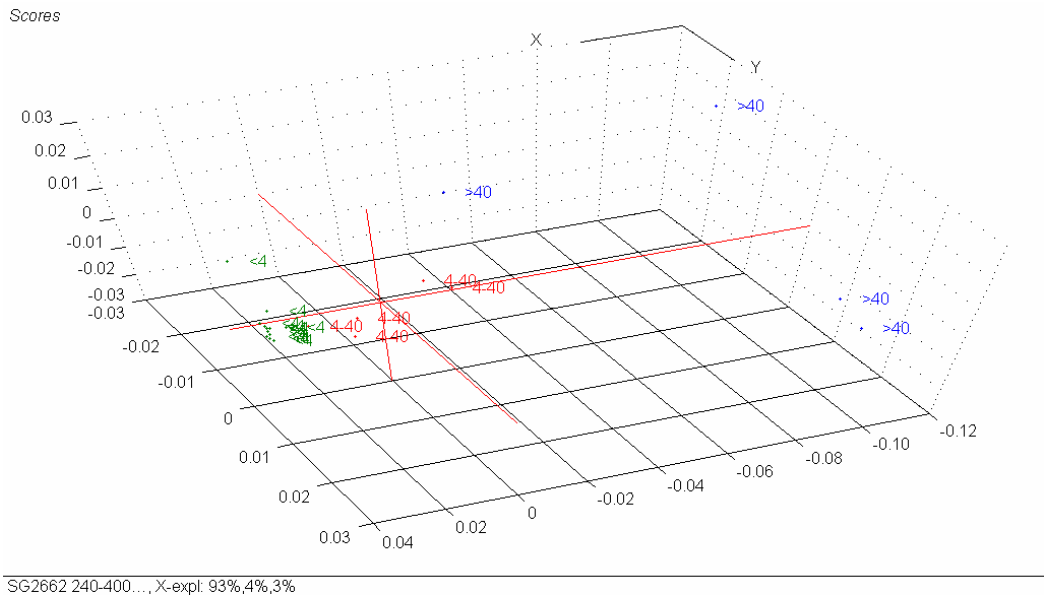


Figure 6.40 A plot of the first 3 principal component scores of second derivative transformed UV spectra (240–400 nm) of extracts of un–inoculated control and *E. lata* cultures. Samples are marked by level of eutypinol: < 4µg/200 µL (green), 4–40 µg/200 µL (red), >40 µg/200 µL (blue).

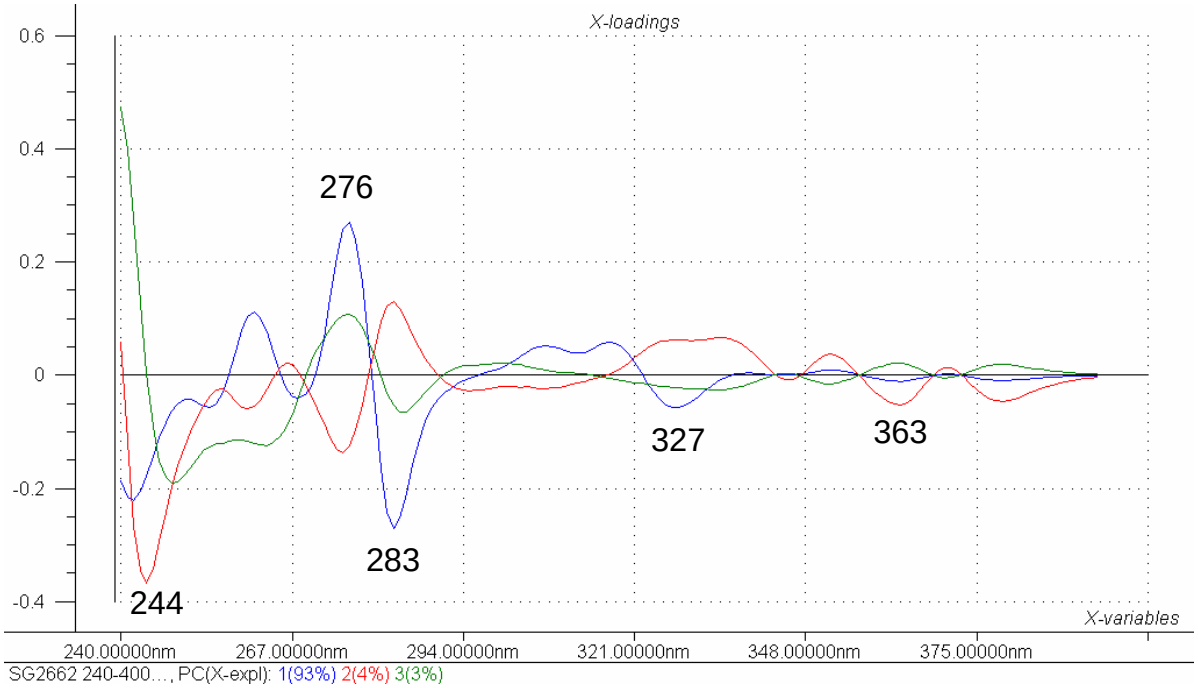


Figure 6.41 Loadings for principal component analysis of second derivative transformed UV spectra (240–400 nm) of extracts of un–inoculated control and *E. lata* cultures.

Finally, linear discriminant analysis (LDA) was performed using the first 9 PCs as the input and eutypinol concentration as the output. The overall gradation of concentration was not even, so samples with a lower concentration of eutypinol tended to overlap (see canonical score plots in Figure 6.42), but high concentration samples were clearly discriminated. The classification rate in training was 95% correct, and 81% correct with 1–out cross–validation: considering the relatively small size of the sample set, this is a sound result in cross–validation.

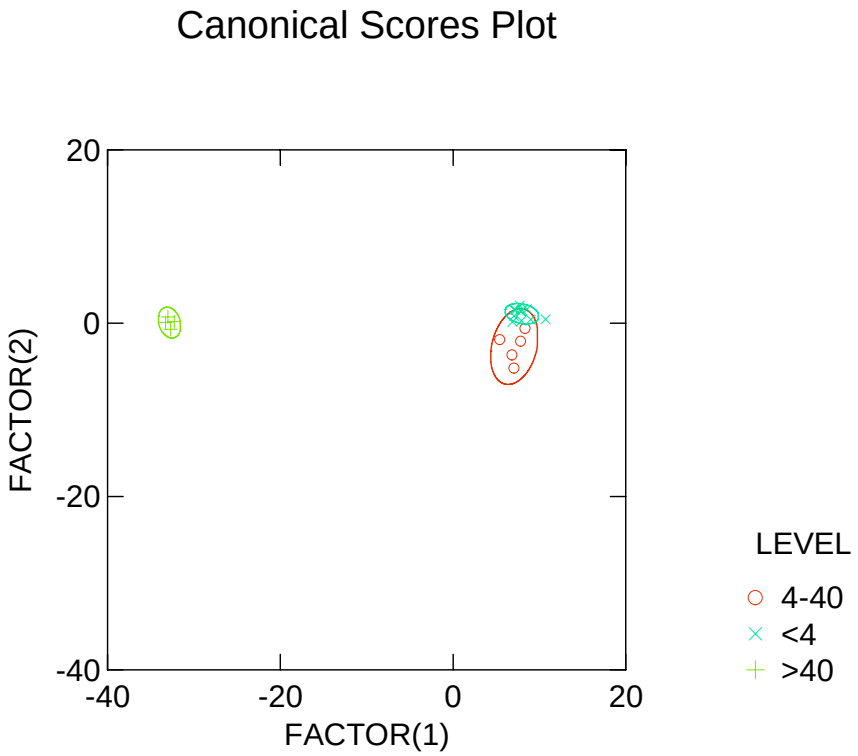


Figure 6.42 Canonical score plot from linear discriminant analysis (LDA) using 9 principal component scores of 2nd derivative UV spectra as the input: 95% correct classification in training, 81% correct with 1–out cross–validation.

Detection of eutypine by Raman spectroscopy and thin layer chromatography

- Raman spectroscopy

The Raman profile of pure eutypine showed a strong peak at 2,204 cm^{-1}. However, analysis of foliage obtained from vines showing symptoms of eutypa dieback did not detect eutypine in either the leaf surface, beveled cut edge of a leaf, longitudinal section of the petiole, or in the stems or sap.

- Thin layer chromatography

Analysis of metabolites produced by 19 isolates of *E. lata* grown in Pezet's culture medium showed that numerous compounds were produced by several isolates after 14 days growth. A metabolite with Rf 0.34 was the most consistently produced. Only two isolates, L003 and M303, produced eutypine (Table 6.10). After 42 days of growth, no eutypine was detected, although five other compounds were present. At 56 days, five isolates produced a total of six metabolites; only isolate L003 produced eutypine. At 70 days, seven isolates produced a total of four metabolites and, again, only isolate L003 produced eutypine.

Table 6.10 Production of eutypine and other metabolites by isolates of *E. lata* over time.

		Days											
		14			42			56			70		
		Rep			Rep			Rep			Rep		
ME	Isolate	1	2	3	1	2	3	1	2	3	1	2	3
1	Control	-	-	-	-	-		-	-	-	-	-	-
2	M303	-	+	-	-	-		-	-	-	-	-	-
3	L003	+	+	+	-	-		-	-	+	-	-	+
4	L001	-	-	-	-	-		-	-	-	-	-	-
5	E100	-	-	-	-	-		-	-	a	-	-	-
6	B001	-	-	-	-	-		-	-	-	-	-	-
7	C002	-	-	-	-	-		-	-	a	-	-	-
8	B010	-	-	-	-	a		-	-	-	a	-	-
9	L002	-	-	-	-	-		-	-	-	-	-	-
10	14/02	-	-	-	-	-		-	-	-	-	-	-
11	D001	-	-	-	-	-		-	-	-	-	-	-
12	E202	-	-	-	-	-		-	a	-	-	a	-
13	273/01	-	-	-	-	-		-	-	-	-	-	-
14	C001	-	-	-	-	-		-	-	-	-	-	-
15	E002	-	-	-	-	-		-	-	-	-	-	-
16	E201	-	-	-	-	-		-	-	-	-	-	-
17	D010	-	-	-	-	-		-	-	-	-	a	-
18	B007	-	-	-	-	a		-	-	-	-	-	-
19	E003	-	-	-	-	a		-	-	-	-	-	-
20	C003	-	-	-	-	-		-	-	-	-	-	-

+ = eutypine present, - = no compounds present, a = compounds other than eutypine present

TLC analysis of cuttings inoculated with *E. lata* detected a compound with Rf matching the eutypine standard in apical leaves of nine of 20 inoculated plants and one of four uninoculated control plants, and in basal leaves of 12 of 18 inoculated plants and two of four uninoculated control plants. Buds failed to burst on ten of the 72 plants inoculated with *E. lata* at Monash University and 16 plants exhibited shortened internodes. However, TLC analysis did not detect eutypine in any of the leaf tissues.

The inability to detect eutypine in foliage of naturally infected vines using Raman spectroscopy may be because the foliage was assessed in autumn, by which time the eutypine may have been degraded to eutypinol or other compounds (Colrat *et al.*, 1999, Molyneux *et al.*, 2002). Furthermore, measurement was limited to the surface of the opaque material and autofluorescence of chlorophyll in the sample may have obscured the Raman signal. The observation that a compound with Rf matching that of eutypine was detected in healthy control vines implies that TLC is not a suitable method for detecting this compound *in planta*.

Influence of temperature and water availability on secondary metabolite production by E. lata

Linear growth rate of *E. lata* was strongly influenced by temperature and matric potential. The average linear growth across all treatments is summarised in Table 6.11 Optimum growth across all treatments

(5.35 mm/day) was achieved at 25°C with a matric potential of –1 MPa; there was a considerable decrease in growth rate at 30°C at this matric potential. However, when grown on media with matric potentials of –2 and –3 MPa, maximum growth was at 30°C (5.10 and 4.64 mm/day, respectively). Further experiments would be required to ascertain optimal temperature for growth in these treatments, given that temperatures used in this experiment did not exceed 30°C. There was a pronounced inhibition of growth when matric potential was decreased to –6 MPa, with maximum growth of 2.54 mm/day (25°C). There was considerable variation in growth rate between isolates. For example, when grown under optimum conditions (25°C, –1 MPa) the average growth rate of each isolate varied from 4.28 to 6.89 mm/day. When incubated at 12.5°C, two isolates showed no growth between days 4 and 7 at a matric potential of –6 MPa; the remaining four isolates exhibited growth at between 0.67 and 1.33 mm/day.

Table 6.11 Average growth rate of six isolates of *E. lata* at each matric potential/temperature combination.

MPa	Average growth rate (mm/day) (S.E.)			
	12.5°C	**17.5°C**	**25°C**	**30°C**
–0.27	1.54 (0.48)	2.14 (0.26)	4.23 (1.28)	1.51 (0.73)
–1.0	2.69 (0.70)	3.39 (0.88)	5.35 (0.95)	2.66 (1.70)
–2.0	1.82 (0.65)	3.13 (0.94)	4.89 (1.08)	5.10 (1.53)
–3.0	1.04 (0.43)	2.06 (0.56)	3.33 (0.52)	4.63 (1.12)
–6.0	0.52 (0.45)	2.18 (1.22)	2.54 (1.38)	2.48 (1.02)

At the time of writing, the full set of data for secondary metabolite production was not available. However, preliminary analysis of isolates grown at 25°C with matric potentials of –0.27, –1, –2 and –3 MPa showed that eutypinol was the predominant metabolite, accounting for between 70 and 90% of total metabolites produced, depending on the matric potential. Similar to growth rate data, optimal metabolite production occurred at a matric potential of –1 MPa, although the interactions between temperature, matric potential and metabolite production have not yet been fully elucidated.

6.4.5.3 Identification of chemical marker(s) in treated and infected vines

Detection of secondary metabolites in micropropagated vines treated with metabolites of E. lata

The excised shoot treated with eulatachromene showed extensive bleaching of the stem and collapse of the plant after 10 days exposure. The shoot treated with eutypine showed some stem and leaf bleaching and the shoot treated with eutypinol exhibited some browning of the stem. Shoots treated with siccayne and ethylene glycol remained healthy. HPLC analysis of treated shoots detected eutypinol and eulatachromene, but not eutypine or siccayne.

Shoots treated with metabolites from isolates E1 and M266 showed bleaching of stems and necrosis of foliage after 10 days; shoots treated with metabolites from the remaining seven isolates were asymptomatic. All plantlets treated with crude MYB filtrate displayed widespread necrosis; this may be due to the pH of the medium, which becomes alkaline following growth of *E. lata*.

Detection of metabolites in treated plant material was variable, and yields were generally small relative to the purified culture filtrate (Table 6.12). Eutypinol could be detected in excised shoots treated with purified culture filtrates, although always in small quantities and only when shoots were treated with extracts containing more than 100 µg of eutypinol. Other compounds, including eulatachromene, benzofuran and eulatinol, were also detected. However, eutypine was not detected in any of the plantlets analysed here. The amount of metabolites recovered was generally less in rooted plantlets treated with crude filtrates than in shoots treated with purified filtrates (Table 6.12).

Table 6.12 Detection of secondary metabolites in plantlets exposed to purified and crude culture filtrates of *E. lata* for 10 days

Isolate	Treatment	Metabolites (µg)									
		Eutypinol	Methyl eutypinol	Eulatachromene	Eutypine	Methyl eutypine	Benzofuran	Eulatinol	Siccayne	rt 16.5 min[a]	rt 19.5 min[a]
E1	Purified filtrate (HPLC)	170	0	0	104	5.3	111	76	36	+	-
	Excised shoot treated with purified filtrate	0.7	0.6	0	0	0	0.9	0.7	0	-	-
	Ethylene glycol solution	77	25	13	0	0.9	46	24	0	+	-
	Rooted plantlet treated with crude filtrate	0	0	0	0	0	0	0.4	0	+	+
	Crude filtrate/roots	0	0	0	0	0	0	25	0	+	+
E120	Purified filtrate (HPLC)	13	0	0	0.6	0	0.2	24	30	+	-
	Excised shoot treated with purified filtrate	0	0	0	0	0	0	0.2	0	-	-
	Ethylene glycol solution	6.9	0.5	0	0	0	0	158	0	-	-
	Rooted plantlet treated with crude filtrate	0	0	0	0	0	0	0.7	0	+	-
	Crude filtrate/roots	2.4	0	0	0	0	0	29	0	+	-
E125	Purified filtrate (HPLC)	203	4.1	7.8	6.1	3.5	10	21	0	+	-
	Excised shoot treated with purified filtrate	0.8	0.7	0	0	0	0	0	0	+	+
	Ethylene glycol solution	93	22	2.7	0	0	0	0	0	+	+
	Rooted plantlet treated with crude filtrate	0	0	0	0	0	0	0	0	+	-
	Crude filtrate/roots	3.7	12	0	0	0	0	35	0	+	+
M266	Purified filtrate (HPLC)	1273	0	58	21	0	3.6	0	0	-	-
	Excised shoot treated with purified filtrate	10	0.3	5.9	0	0	0.4	0	0	-	-
	Ethylene glycol solution	409	0	31	13	0	2.8	0	0	+	-
	Rooted plantlet treated with crude filtrate	0.2	0	0	0	0	0	0	0	+	-
	Crude filtrate/roots	36	0	0	12	0	5.6	0	0	+	+
M279	Purified filtrate (HPLC)	1.3	0.9	0.4	0	0.3	0.8	0.6	0	+	-
	Excised shoot treated with purified filtrate	0	0	0	0	0	0	0	0	-	-
	Ethylene glycol solution	0	0	0	0	0	0	0	0	-	-
	Rooted plantlet treated with crude filtrate	0	0	0	0	0	0	0	0	-	-
	Crude filtrate/roots	0	0	0	0	0	0	0	0	-	-
M280	Purified filtrate (HPLC)	327	0	6	3.2	0	2.6	66	0	-	-
	Excised shoot treated with	0.4	0	0	0	0	0	2.7	0	-	-

Isolate	Treatment	Metabolites (µg)									
		Eutypinol	Methyl eutypinol	Eulatachromene	Eutypine	Methyl eutypine	Benzofuran	Eulatinol	Siccayne	rt 16.5 min[a]	rt 19.5 min[a]
	purified filtrate										
	Ethylene glycol solution	5.2	0.3	0	0	0	0	14	6.4	-	-
	Rooted plantlet treated with crude filtrate	0	0	0	0	0	0	1	0	-	-
	Crude filtrate/roots	0	0	0	0	0	0	41	0	-	-
M335	Purified filtrate (HPLC)	0	0	0	0	0	0	0	0	-	-
	Excised shoot treated with purified filtrate	0	0	0	0	0	0	0	0	-	-
	Ethylene glycol solution	0	0	0	0	0	0	0	0	-	-
	Rooted plantlet treated with crude filtrate	0	0	0	0	0	0	0	0	-	-
	Crude filtrate/roots	0	0	0	0	0	0	0	0	-	-
N04	Purified filtrate (HPLC)	269	149	7	0	1.6	2.7	78	0	+	-
	Excised shoot treated with purified filtrate	2.4	2.7	0	0	0	0.1	1.3	0	-	+
	Ethylene glycol solution	148	81	6.3	0	1	5.5	45	0	+	-
	Rooted plantlet treated with crude filtrate	0.5	0.6	0	0	0	0.3	1.1	0	+	+
	Crude filtrate/roots	26	18	0	19	1.6	3.6	59	0	+	+
SS6	Purified filtrate (HPLC)	0	1.2	2.5	0.3	1.3	0.4	34	2.8	-	-
	Excised shoot treated with purified filtrate	0	0	0	0	0	0	0.5	0	-	-
	Ethylene glycol solution	0	1	0	0	0	0	11	1.1	-	-
	Rooted plantlet treated with crude filtrate	0	0	0	0	0	0	0.6	0	-	-
	Crude filtrate/roots	0	0	0	0	0	0	28	1.9	-	-

= not detected

[a] Quantitative data for metabolites with retention times of 16.5 and 19.5 min could not be determined as these compounds have not been fully characterised. Presence of these compounds is therefore indicated by '+', and absence by '-'.

Two novel compounds, with retention times of 16.5 and 19.5 min, were also detected. These compounds, tentatively identified as eutypinic acid and methyl eutypinic acid, were commonly present at higher levels in treated shoots and plantlets than in the purified culture filtrate analysed by HPLC; the compound with a retention time of 19.5 min was not present in purified culture filtrates, but was detected in excised shoots treated with purified filtrates and in plantlets treated with crude filtrates.

Analysis of purified filtrates from isolates used in the time course experiment showed that eight compounds were present in amounts ranging between 0.1 and 234 μg (data not shown). However, uptake of metabolites by micropropagated shoots treated with these extracts was apparently very low and no compounds were consistently detected in all samples. Furthermore, there was no apparent correlation between the amount and type of metabolite detected and the period of exposure to culture filtrates. Because of the large scale of the experiment we were unable to repeat this trial.

Analysis of plantlets treated with pure metabolites of *E. lata* showed that two of four compounds could be detected 10 days after treatment. Because of difficulties in obtaining sufficient quantities of individual metabolites, these experiments could not be replicated, and nor were any other metabolites of *E. lata* tested. Analysis of micropropagated plantlets treated with culture filtrates showed low yields of metabolites. Although eutypinol was detected in excised shoots treated with purified culture filtrates, it was detected less consistently in intact plantlets treated with crude culture filtrates. This may be due to poor uptake of compounds by rooted plantlets, however, other compounds, in particular eulatinol, could be detected in rooted plantlets and in excised shoots. This implies that failure to detect some compounds may be caused by other factors, such as degradation of compounds when in aqueous solution, or perhaps by *in planta* conversion to compounds not detected using HPLC.

The detection of previously unidentified metabolites in treated plantlets, which were absent or present in very small amounts in purified culture filtrates, provides some evidence for the transformation of metabolites *in planta*. It was anticipated that the time course experiment would generate information on the longevity and degradation of metabolites following entry into plant tissue. However, for reasons which remain unclear, there was very little uptake of metabolites in this experiment, and very little information was gathered. Because other compounds such as methyl eutypinol and the benzofuran compound could be detected in treated plantlets, even when present in smaller amounts than eutypinol in the purified culture filtrates analysed by HPLC, the possibility of using these compounds as chemical markers may warrant further investigation.

Detection of metabolites in micropropagated vines inoculated with E. lata

Expression of foliar symptoms was inconsistent in inoculated material, with few plants showing typical symptoms of eutypa dieback. Most plants were asymptomatic, or became overgrown with mycelium that totally covered the plant and growth media. HPLC analysis of plant extracts detected eutypinol in some, but not all, symptomatic plantlets and in some inoculated, asymptomatic material. No other secondary metabolites characteristic of *E. lata* were detected. The other protocols tested here did not enable the reliable induction of foliar symptoms in micropropagated material, and neither did using the more susceptible cultivar, Grenache. HPLC analysis did not consistently detect any metabolites characteristic of *E. lata* in these experiments.

The detection of eutypinol in asymptomatic plantlets supported the hypothesis that metabolites of *E. lata* may be present in infected vines before foliar symptoms become visible. However, neither eutypinol nor any other metabolites were consistently detected in inoculated plantlets. It remains unclear why we were unable to induce symptoms of eutypa dieback reliably on micropropagated plantlets. One explanation may be that the non–lignified tissue of micropropagated plantlets is not an appropriate substrate for the production of toxic metabolites.

HPLC analysis of extracts from inoculated and naturally infected vines

HPLC analysis of extracts from the foliage of naturally infected vines in the field did not identify any metabolites characteristic of *E. lata*, regardless of isolation protocol. Similarly, HPLC analysis did not detect any metabolites characteristic of *E. lata* in sap extracted from symptomatic and asymptomatic vines using a pressure chamber.

The inability to detect any metabolites in the sap of infected vines using HPLC implies that these metabolites may not be suitable chemical markers for diagnosing eutypa dieback. This was supported by the failure to detect metabolites using other techniques including Raman spectroscopy and TLC. The observation by Mahoney *et al.* (2005) that metabolites of *E. lata* could not be detected in naturally infected vines showing foliar symptoms of eutypa dieback also indicates that these metabolites may not be suitable chemical markers. Reasons for this remain unclear; it is possible that, following their entry into grapevine tissue, such metabolites are rapidly broken down into compounds that cannot be detected using HPLC. Alternatively, the suggestion of Mahoney *et al.* (2005) that such metabolites are not translocated from the site of infection may explain our inability to detect these compounds *in planta*. Octave *et al.* (2006) isolated polypeptide compounds from a single isolate of *E. lata* grown in artificial culture and showed that mesophyll cells in leaves treated with such compounds underwent modifications similar to those observed in naturally infected vines. Hence, it is possible that such compounds may prove a reliable indicator of infection by *E. lata*. However, before such research is initiated it would be necessary to confirm that these polypeptides are produced by the majority of isolates of *E. lata*, and that they are not an artefact of the medium upon which the fungus is grown.

Results of the preliminary research on secondary metabolite production were published in Phytochemistry (Mahoney *et al.*, 2003) and results of subsequent experiments have been accepted for publication in the Australian Journal of Grape and Wine Research (Lardner *et al.*, in press). Copies of these manuscripts are included in Appendix 1.

7 OUTCOMES AND CONCLUSIONS

7.1 Epidemiology

Output 1: Epidemiological studies of *E. lata.*
Performance Targets:

- Growth rate of *E. lata* quantified in selected grapevine cultivars by isolation and DNA–based techniques.
- Symptom development compared in selected grapevine cultivars.
- Underlying cause of annual variation in symptom expression determined, if possible.
- The rate of disease progress over consecutive years determined to identify when remedial action should be taken and/or when vineyards should be replanted.

All performance targets relating to epidemiology were achieved. Research has provided new methodologies for studying host–pathogen interactions. Results will enhance the management of eutypa dieback and help grapegrowers achieve increased economic returns through improved yield and quality.

Results from ascospore release studies have led to recommendations that growers avoid practices which expose wounds to the air, such as pruning, desiccating or reworking, during or immediately following rainfall in excess of 1 mm. This will reduce the potential for infection.

The growth rate of *E. lata* in selected cultivars was quantified by isolation and DNA analysis. In mature vines in the field, the mean spread of staining due to *E. lata* differed significantly among cultivars, ranging from 10 mm/yr for cvs Merlot, Gamay, Grenache and Semillon to 18 mm/yr for cvs Cabernet and Shiraz. However, it is important to note that there was some variation and, in a few cases, the staining progressed as much as 50 mm/yr in cvs Cabernet and Shiraz. On younger vines in the shadehouse, the mean growth rate of *E. lata* in Cabernet Sauvignon, Merlot and Grenache was 15, 13 and 18 mm/yr, respectively, with a maximum rate of 115 mm/yr recorded on Grenache. The staining of wood typically associated with eutypa dieback did not always correlate with the presence of the fungus and *E. lata* grew up to 80 mm in advance of staining in young vines. The *E. lata*–specific probe proved to be a reliable, sensitive and efficient means of detecting the fungus in wood.

Foliar symptoms developed on young grapevine cuttings within 8 months of inoculation with mycelium in a shadehouse. Twenty–six of the 40 isolates tested induced foliar symptoms in the first spring and, within 20 months of inoculation, 31 isolates induced symptoms. Geographical origin of isolates appeared to influence foliar symptom severity. Development of foliar symptoms was significantly greater in Grenache than in Cabernet Sauvignon or Merlot. Mature vines artificially inoculated with ascospores in the field did not develop foliar symptoms within 4 years of inoculation. The shadehouse bioassay developed in this project provides a rapid and effective technique for further studies.

Foliar symptoms of eutypa dieback fluctuate from year to year in vineyards, causing variation in crop yield and value. It is likely that environmental factors such as climate play a role in this phenomenon. According to our preliminary analysis, rainfall and temperature may influence severity and incidence of foliar symptoms of eutypa dieback. Rainfall was also linked to the infection of pruning wounds and may affect concentration or movement of toxins in the vine through water uptake. Temperature affects vine vigour and may also affect the ability of the fungus to produce toxins. These relationships and possible causes require further examination to confirm the causes of symptom variation.

Due to the slow progress of eutypa dieback in grapevines, it has typically been difficult to establish when remedial action should be taken. Knowledge that colonisation of wood progresses at up to 50 mm/yr gives growers an indication of the progress of infection along cordons so that infected wood may be removed before it reaches the trunk, or, in advanced cases, before it reaches ground level, after which little can be done to revive the vine.

7.2 Prevention

Output 2: Develop biological and chemical control strategies for eutypa dieback.
Performance Targets:

- Wound protection treatments identified that can be applied effectively using commercial spray equipment within several days of pruning, that provide long–lasting protection, and that may also have curative properties.
- The efficacy of wound protection treatments applied to large wounds, i.e. wounds greater than 20 mm in diameter, established.
- The efficacy of wound protection treatments applied in 2001 to 20–year–old Sauvignon Blanc vines when top–worked to Shiraz assessed.

Numerous trials were undertaken to identify products which will control eutypa dieback. Of the fungicides, Benlate (benomyl) and Bavistin (carbendazim) were most effective in preventing infection of wounds. Because Benlate and all benomyl products have been withdrawn from the market, steps have been taken to extend the label registration of Bavistin for use on grapevine wounds to control eutypa dieback. Other fungicides, such as Cabrio (pyraclostrobin), BASF 516 (boscalid + pyraclostrobin), Nustar (flusilazole), Shirlan (fluazinam), Scala (pyrimethanil) and Fungaflor (imazalil) require more extensive evaluation to ascertain if registration to control of eutypa dieback is warranted. The *E. lata* specific SCAR marker, once validated, was used routinely to verify that inoculum was indeed *E. lata.*

Application of treatments using commercial spray equipment would reduce the time involved in protecting pruning wounds and hence the cost. Results of preliminary trials showed that spray application can be as effective as application with a paint brush. Preliminary results from INRA, France show that spraying fungicides onto vines with a recycling system is as effective as painting, and more cost effective (Lecomte *et al.*, 2003).

Paints and pastes proved effective alternatives to fungicides mixed in water. Acrylic paint prevented infection by *E. lata*. Several paste products, such as Garrison and Nectec, which contain fungicides, were also effective in the prevention of infection. One of the reasons for the success of paints and pastes is likely to be due to the physical barrier they provide against entry of ascospores into fresh pruning wounds. Chemcolour Industries is currently considering registration of Garrison for use on grapevines for eutypa dieback on the basis of results obtained from trials in this project. Paints and pastes are limited to application by hand but are recommended to growers when conducting drastic pruning or reworking of vines.

Trichoseal (*Trichoderma* spp.) was not effective in these trials, however, John *et al.* (2005b) reported that application of *Trichoderma* spp. and products to wounds reduced infection by *E. lata*. Further testing is necessary to determine the reasons for the variation and hence the conditions necessary for effective control of the disease using this biological agent.

The efficacy of pruning wound treatments on larger wounds could not be determined due to infrequent infection of inoculated controls or insignificant effects of treatments, possibly due to pre-existing *E. lata* infection.

Wound treatments on grafted vines did not affect watershoot production or recurrence of foliar symptoms. It is most likely that Bitkote sealant provided a physical barrier to infection, suggesting that treatment with fungicides was unnecessary in this situation.

7.3 Control

Output 3: Evaluate curative/fungistatic treatments to restore productivity to Eutypa–infected vines.
Performance Targets:

- Efficacy of novel products for the treatment of infected vines assessed.
- The most effective method of conducting remedial surgery determined.
- The short– and long–term effects of remedial surgery in removing infected tissue and restoring vineyard productivity and longevity determined.

The efficacy of several novel products to control infected vines was assessed. Injection of fungicides had no influence on vine yield or the development of eutypa dieback symptoms. *Trichoderma* products, either injected or inserted via dowels into trunks, did not improve yields or alleviate eutypa dieback symptoms compared to untreated vines.

Foliar application of Brotomax, a nitrogen–based foliar fertilizer, increased yields by 20% in severely diseased vines after 3 years of regular application. However, yield increase was not apparent in the fourth year, when conditions were milder and wetter than previous years. Although no cost–benefit analysis has been performed, it is recommended that growers consider this method only if vines are likely to be under stress and yield increase is desired. However, this method cannot be recommended for the control of eutypa dieback in grapevine.

Remedial surgery to restore eutypa dieback–affected vines to productivity is a labour intensive and costly process. This research has demonstrated the short–term effectiveness of remedial surgery as a strategy for managing eutypa dieback–infected vines. The success of the two methods evaluated was affected by cultivar and the extent of infection. Information on longer–term effects of remedial surgery, requires assessment over the next 5–10 years.

Growers are currently advised to monitor vines in early spring for foliar symptoms and to tag symptomatic cordons in preparation for removal by remedial surgery in winter. We have shown that foliar symptoms are not always associated with wood infection. Therefore, it is unlikely that removal of vine parts that are associated only with foliar symptoms will guarantee removal of all infected tissue from a vine. As foliar symptom expression varies from year to year, monitoring foliar symptoms for several seasons will increase the chances of locating infected vines.

Remedial surgery aims to remove all diseased wood from a vine. However, it is possible that mycelium of *E. lata* can colonise new wood, but not manifest symptoms for several years. If the pathogen is not completely removed from the vines, longevity of the reworked vines may be limited. Results from this project have confirmed the recommendation that, when removing stained tissue, growers should cut back a further 10 cm or more to ensure removal of any mycelium of *E. lata* in advance of the staining.

As *E. lata* infects through wounds, it is important to protect all wounds resulting from remedial surgery. The application of acrylic paint, paste or an effective fungicide, such as Bavistin, to wounds will reduce the likelihood of new infection of the vine by *E. lata*.

7.4 Diagnosis

Output 4: Information on chemical marker(s) for use as a diagnostic marker for *E. lata*.
Performance Targets:

- Identification and characterisation, by HPLC and LCMS, of unique chemical marker(s) consistently produced by *E. lata* in pure culture.
- Evaluation of NIR as a means of rapid detection of chemical marker(s) in extracts of pure cultures of *E. lata*.

Eutypinol was produced by all but one isolate of *E. lata* in pure culture and was identified as a suitable chemical marker for the disease. Grapevine–based media were identified as particularly appropriate for assessing secondary metabolite production by *E. lata*. When grown on grapevine media, several other compounds, including methyl eutypinol and benzofuran, were produced by over 80% of isolates; these compounds may also be suitable chemical markers. We showed that the phytotoxin eutypine was not produced by all isolates of *E. lata*; this compound was produced by 76% of isolates, in smaller amounts than the compounds identified above. Analysis of secondary metabolite production by other grapevine–inhabiting fungi showed that none produced any metabolites in common with *E. lata*, confirming the suitability of the above compounds as specific markers for *Eutypa* infection.

Discussions with Dr Bob Dambergs (AWRI) concluded that UV–Vis spectroscopy was more suited to identifying *Eutypa* metabolites than NIR, given the structure of the metabolites involved. The preliminary study showed, that in pure culture filtrates, UV–Vis spectroscopy clearly identified metabolites of *E. lata*, in particular eutypinol. Furthermore, the technique differentiated between un–inoculated control samples and samples of *E. lata* which produced no metabolites; this was not possible using HPLC.

Output 5: Information on detection of chemical marker(s) in extracts or ground foliage of treated and inoculated vines.
Performance Targets:

- Evaluation of HPLC, LCMS and/or NIR for detection of chemical marker(s) in foliage of vines injected with the chemical marker(s).
- Evaluation of HPLC, LCMS and/or NIR for detection of chemical marker(s) in foliage of vines inoculated with *E. lata* in tissue culture and in the glasshouse.

Initial experiments showed that eutypinol and eulatachromene, but not siccayne or eutypine, could be detected by HPLC in micropropagated shoots treated with individual metabolites. Similarly, some, but not all, metabolites present in purified culture filtrates of *E. lata* could be detected in material treated with these filtrates. Once again, eutypinol and eulatachromene could be detected, but not siccayne or eutypine. In addition, two previously unidentified compounds, one of which was present only after grapevine tissue was exposed to culture filtrates, were detected. Using LCMS, these compounds were tentatively identified as eutypinic acid and methyl eutypinic acid. Amounts of compounds detected were generally small relative to the amount in the pure filtrates with which shoots were treated.

Inoculation of micropropagated vines with mycelium of *E. lata* showed that symptoms were difficult to induce. However, HPLC analysis of inoculated vines did detect eutypinol, but no other compounds, in some symptomatic and some asymptomatic vines. This supported the hypothesis that metabolites of *E. lata* may be present in the foliage before disease symptoms became visible.

HPLC analysis of vines in a shadehouse showed that neither eutypinol nor any other acetylenic phenol compounds could be detected in symptomatic or asymptomatic vines. Likewise, analysis of artificially inoculated material using Raman spectroscopy and TLC also failed to detect metabolites characteristic of *E. lata*.

Output 6: Information on detection of chemical marker(s) in foliage of naturally infected vines.
Performance Targets:

- Evaluation of HPLC, LCMS and/or NIR for detection of chemical marker(s) in foliage of naturally infected vines.

- Comparison of spectra for foliar extracts and whole leaves, using lab–based NIR and hand–held radiometer.

HPLC and Raman spectroscopy were used to detect chemical markers in the foliage of naturally infected vines. However, even when samples were taken in the spring, when metabolites are expected to be most abundant, we were unable to detect any fungal metabolites in vines displaying foliar symptoms of eutypa dieback. These results were confirmed in an independent series of experiments conducted in the laboratory of Dr Russell Molyneux (USDA), who also investigated the possibility that metabolites of *E. lata* may be glycosylated and, hence, not extracted with organic solvents, and showed that this was not the case.

Because metabolites of *E. lata* could not be detected in foliar extracts, spectra for foliar extracts and whole leaves were not compared.

Output 7: Validation of chemical marker(s) as an indicator of infection using DNA–based probe.
Performance Target:
- Application of DNA–based marker to confirm the presence of *E. lata* in naturally infected vines.

Because chemical markers could not be detected in naturally infected vines, DNA markers were not used for this purpose, although they were widely used in other parts of the study.

Output 8: Information on response of vines to chemical and biological treatments.
Performance Target:
- Use chemical markers and spectroscopic techniques to monitor the response of vines to treatment with chemical or biological control agents identified above.

Because chemical markers could not be detected in infected vines, this output was not achieved.

8 RECOMMENDATIONS

8.1 Epidemiology

Effective management of eutypa dieback relies on understanding the epidemiology of *E. lata*. This research has significantly improved our knowledge and provided important management information for growers; it has also highlighted areas where further research is needed.

Ascospore release is an important part of the disease cycle and further research into the amount of rainfall required before stromata are depleted of ascospores and the length of time before stromata are replenished is required. Likewise, the effect of cultivar, time of year and region on the release of *E. lata* ascospores requires further study.

New information about the relationship between foliar symptoms and climate has provided several hypotheses about the influence of environmental conditions on the expression of foliar symptoms. Experiments should be designed to test these hypotheses, initially using rooted vine cuttings in the glasshouse or growth room in controlled conditions of soil moisture and temperature. Field trials should also be conducted on vines subjected to a range of irrigation systems to determine the effect of water stress on infection and disease progression over a long period of time. Use of DNA and biochemical diagnostic techniques (Lardner *et al.* 2004) will provide quick and accurate results. Continued monitoring of the vineyards studies here and of vineyards in other regions over 5–10 years would allow us to confirm or refine the conceptual model which predicts the incidence of foliar symptoms in eutypa dieback–affected vineyards. Information obtained could be incorporated into an integrated pest management system utilising irrigation strategy to suppress foliar symptoms of eutypa dieback. Combined with preventative and control strategies, this could maximise productivity and profitability of grapevines in eutypa dieback–affected vineyards.

The shadehouse bioassay provides a new method to; (i) screen cultivars of grapevine for susceptibility to eutypa dieback, (ii) investigate the mechanisms by which cultivars such as Merlot tolerate infection by *E. lata* and (iii) investigate the effects of moisture, climate and nutrition on foliar symptoms and fungal colonisation.

Botryosphaeria spp., not *E. lata*, were responsible for dieback of vines in the Hunter Valley and Mudgee regions of NSW, but further work is now required to determine; (i) the species of *Botryosphaeria* responsible, (ii) the occurrence and impact of Botryosphaeria dieback in Australia, (iii) whether there is a climatic divide between the occurrence of eutypa dieback and Botryosphaeria dieback and (iv) the epidemiology of the diseases.

Eutypa dieback is not considered a problem in emerging wine regions, such as the Adelaide Hills, Fleurieu Peninsula, Mt Benson and Robe in South Australia or in Western Australia and Tasmania, so little preventative action is being taken. The presence and extent of the disease in these regions needs to be determined and extension programs undertaken to alert growers to adopt preventative strategies to avoid yield and quality loss and increase the sustainability of vineyards.

8.2 Prevention

Bavistin (carbendazim) is an effective alternative to Benlate (benomyl) for protecting wounds from infection by *E. lata*. However, the registration of several benzimidazole fungicides is under review and effective products may be removed from the market in the future. Also, *E. lata* may become resistant to these single–site fungicides. A range of alternative fungicides should be identified. Cabrio (pyraclostrobin), BASF 516 (boscalid + pyraclostrobin), Nustar (flusilazole), Shirlan (fluazinam), Scala (pyrimethanil) and Fungaflor (imazalil) were effective in preliminary trials and require further testing to confirm their ability to provide long–lasting protection from infection by *E. lata*.

Research in South Africa, France and Greece has identified carbendazim, flusilazole and fluazinam as suitable wound protectants. Therefore, it is recommended that both flusilazole and fluazinam are prioritised for further evaluation in the future.

Mechanisation is crucial to the economic viability of large–scale vineyard management in Australia. Therefore, wound protectants need to be applied by commercial sprayers. Preliminary results indicated that spray application is a viable prospect and future research aims to develop efficient application systems.

Further research is required to identify treatments for large wounds caused when trunks are cut during remedial surgery (i.e. wound diameter larger than 5 cm).

DNA based markers should be used in all future experiments to verify the identity of ascospore inoculum and confirm ambiguous cultures as *E. lata* via the SCAR marker in PCR. The use of the *E. lata*–specific DNA probe and slot blot assay to detect *E. lata* in wood, after some refinement to improve cost– and time–efficiency, will also be vital to ensure accuracy of results in future management trials, as the ascospores and mycelium of *E. lata* are readily confused with morphologically similar wood–inhabiting fungi.

8.3 Control

Several novel products failed to alleviate foliar symptoms on eutypa dieback–affected vines. It is possible that such products could be introduced into the trunk of vines with little or no previous infection, as preventative treatments. For example, John *et al.* (2005a) showed that *Trichoderma* could colonise grapevine wood from spore–impregnated dowels inserted into trunks. Vines (Gamay and Pinot Noir) at the Nuriootpa Research Centre, with no symptoms of eutypa dieback, were treated with Vinevax biodowels (*Trichoderma* spp.) in September 2004. These should be challenged by *E. lata* to assess the efficacy of the biodowels as a preventative strategy.

New products, such as Primaxa Stem-Shot, are being marketed for control of eutypa dieback and require independent evaluation. The bioassay developed in this project provides a method to assess such products on *E. lata* in a controlled environment.

Remedial surgery has been used for the management of eutypa dieback–affected vines, but success is dependent on the extent of infection. Short–term results are positive and there is anecdotal evidence that in the long–term, yield and wine quality may improve. Monitoring remedial surgery trials over 10 or more years will help us to determine the most effective techniques for extending the productivity of eutypa–affected vines. Cost–benefit analysis of the process, including quality, would provide growers with more information to aid in decision making for remedial surgery.

8.4 Diagnosis

The slot blot assays provide a reliable means of detecting *E. lata* in infected vines, although this is a relatively time–consuming and costly technique and involves the use of radioactive isotopes. Future research should focus on developing high–throughput DNA extraction protocols which would provide nucleic acid suitable for use in automated analysis of plant material using fluorescence–based PCR.

Reasons for the inability to detect acetylenic metabolites of *E. lata in planta* remain unclear. Results of this and other studies (Octave et al. 2006) provide preliminary evidence that another class of compound may be partially responsible for the foliar symptoms of eutypa dieback. Because early diagnosis of eutypa dieback could minimise losses caused by the disease and enhance management of vines already infected with the pathogen, it is recommended that future research focuses on identifying these other compounds and determining if they can be detected at a distance from the site of infection. An enhanced understanding of the environmental conditions under which such compounds are produced would also improve prospects for successful management of vines already infected with *E. lata*.

Further research should be conducted using crude culture filtrates of *E. lata* and extracts from vines known to be infected with *E. lata* to further assess the suitability of this technique for rapid detection *in vivo*. Scanning of whole leaves using chemical imaging methods should also be assessed to see if this can be used to detect characteristic compounds in infected vines.

APPENDIX 1: COMMUNICATION

Appendix 1.1. Communication within the project

Staff on the project were employed by five organizations located at two sites in Australia and one site in California, USA. The project was also a part of project CRCV 2.2.1 "Managing grapevine trunk diseases" which consisted staff from three organisations. Operational group meetings for staff located at the Waite Campus in Adelaide were held regularly (usually quarterly) throughout the project to plan and organise R&D activities. In most years, meetings were held annually between staff from project CRCV 2.2.1 to discuss project–related issues and review R&D plans and progress either in Adelaide or Melbourne.

Appendix 1.2. Communication with project stakeholders (including CRCV, GWRDC, R&D agencies and industry reference groups)

Reports were prepared regularly for project stakeholders. These included progress and annual reports for CRCV and GWRDC). Presentations and notes on the progress and outcomes of work in the project were given to representatives of CRCV, GWRDC and industry at CRCV Program 2 Review Meetings held on 5 September 2000, 22 May 2001, 10–11 September 2003 and 12 August 2005, and at the CRCV Symposium held on 6–8 June 2005 (see Appendix 1.5). R&D activities in the project also involved regular interaction between project staff and staff of key industry organizations at each location.

Appendix 1.3. Communication with industry and scientific audiences

Information about the progress and outcomes of R&D on eutypa dieback management and diagnosis was communicated to scientific and industry audiences through a series of publications and presentations (see Appendix 1.4 and 1.5).

Appendix 1.4. Publications

Publications relating to work on the project include those listed below. Information on the project was incorporated into reports for project stakeholders and industry, into articles in scientific and industry journals and into papers in proceedings of scientific and industry conferences and workshops.

Scientific publications

Creaser, M.L. and Wicks, T.J. (2001) Yearly variation in eutypa dieback symptoms and the effect on grapevine yield. *Phytopathologia Mediterranea* **40**, S483.

Mahoney, N., Lardner, R., Molyneux, R.J., Scott, E.S., Smith L.R. and Schoch, T.K. (2003) Phenolic and heterocyclic metabolite profiles of the grapevine pathogen *Eutypa lata*. *Phytochemistry* **64**, 475–484.

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

John, S., Scott, E.S., Wicks, T.J. and Hunt, J.S. (2004) Interactions between *Eutypa lata* and *Trichoderma harzianum*. *Phytopathologia Mediterranea* **43**, 95–104.

Mostert, L., Halleen, F., Creaser, M.L. and Crous, P.W. (2004) *Cryptovalsa ampelina*, a forgotten shoot and cane pathogen of grapevines. *Australasian Plant Pathology* **33**, 295–299.

John, S., Wicks, T.J., Hunt, J.S., Lorimer, M., Oakey, H. and Scott, E.S. (2005) Protection of grapevine pruning wounds from infection by *Eutypa lata* using *Trichoderma harzianum* and *Fusarium lateritium*. *Australasian Plant Pathology* **34**, 569–575.

John, S., Malone, J., Scott, E.S., Wicks, T.J. and Hunt, J.S. (2005) Biological control of eutypa dieback in Australia: field evaluation of fungal antagonists. *Phytopathologia Mediterranea* **44**, 113.

Lardner, R., Scott, E.S. and Stummer, B.E. (2006) Genetic variation in Australian isolates of the grapevine pathogen *Eutypa lata*. *Australasian Plant Pathology* (submitted for review).

Lardner, R., Stummer, B.E., Sosnowski, M.R. and Scott, E.S. (2005) Molecular identification and detection of *Eutypa lata* in grapevine. *Mycological Research* **109**, 799–808.

Lardner, R., Mahoney, N., Scott, E. and Molyneux, R. (2005) Biochemical detection of *Eutypa lata* in grapevines. *Phytopathologia Mediterranea* **44**, 93–94.

Lardner, R., Stummer, B. and Scott, E. (2005) Molecular diagnostic tools for the detection of *Eutypa lata* in grapevines. *Phytopathologia Mediterranea* **44**, 94.

Lardner, R., Mahoney, N., Zanker, T.P., Molyneux, R.J. and Scott, E.S. (2006) Analysis of secondary metabolite production by *Eutypa lata* on grapevine extracts and detection of these metabolites *in planta*. *Australian Journal of Grape and Wine Research* (in press).

Lardner, R., Mahoney, N., Zanker, T.P., Molyneux, R.J. and Scott, E.S. (2006) Secondary metabolite production by the fungal pathogen *Eutypa lata*: Analysis of extracts from grapevine cultures and detection of those metabolites *in planta*. *Australian Journal of Grape and Wine Research* **12**, 107–114.

Molyneux, R.J., Mahoney, N., Smith, L.R., Rolshausen, P.E., Lardner, R. and Scott, E. (2005) Diagnosis of *Eutypa* infection by metabolite analysis. *Phytopathologia Mediterranea* **44**, 87.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Effect of Brotomax™ applied to eutypa dieback–affected grapevines in South Australia. *Phytopathologia Mediterranea* **44**, 116–117.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Managing grapevines infected with eutypa dieback using remedial surgery. *Phytopathologia Mediterranea* **44**, 117.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Field evaluation of pruning wound treatments for control of eutypa dieback. *Phytopathologia Mediterranea* **44**, 114.

Sosnowski, M.R., Creaser, M.L. and Wicks, T.J. Protection of grapevine wounds to control infection by *Eutypa lata*. *Plant Disease* (in preparation).

Sosnowski, M.R., Lardner, R., Wicks, T.J. and Scott, E.S. Spread of eutypa dieback within grapevines is influenced by cultivar and isolates of *Eutypa lata*. *Plant Pathology* (in preparation).

Sosnowski, M.R., Shtienberg, D., Creaser, M.L., Lardner, R., Wicks, T.J. and Scott, E.S. Relationships between climate and foliar symptoms of eutypa dieback in grapevines. *Phytopathology* (in preparation).

Industry journals and other media

Creaser, M.L. and Wicks, T.J. (2000) Eutypa dieback: current status and future directions. Australian Grapegrower and Winemaker **438a**, 82–87.

Creaser, M.L and Wicks, T.J. (2000) Eutypa dieback (Revised) In IPM Viticulture: Research to Practice® Training Manual. D. Braybrook, A. Shanks and D. Aitken (Eds.). 4th edition. Cooperative Research Centre for Viticulture, Knoxfield.

Creaser, M.L. and Wicks, T.J. (2001) Yearly variation in eutypa dieback symptoms and the relationship to grapevine yield. Australian Grapegrower and Winemaker **452**, 50–52.

Creaser, M.L. and Raphael, S. (2001) CRCV researchers attend the 2nd International Grapevine Trunk Disease Workshop in Lisbon. CRCV Spring 2001 Newsletter Vol. **8**.

John, S., Lardner, R., Scott, E., Stummer, B. and Wicks T. (2001) Eutypa dieback: research on biological control and diagnostics. Australian Grapegrower and Winemaker **449a**, 73–75.

Creaser, M.L. (2002) Remedial surgery to manage eutypa dieback – not something to rush into. Viticare Newsletter **3** (7).

Creaser, M.L and Wicks, T.J. (2002) Eutypa dieback (Updated) In IPM Viticulture: Research to Practice® Training Manual. D. Braybrook, A. Shanks and D. Aitken (Eds.). 5th edition. Cooperative Research Centre for Viticulture, Knoxfield.

Creaser, M.L. and Wicks, T.J. (2002) Evaluation of fungicides, paints and a biological product for the prevention of eutypa dieback of grapevines. The Australian & New Zealand Grapegrower & Winemaker. **465,** 15-18.

Creaser, M.L. and Wicks, T.J. (2002) Infections extend below ground in vines severely affected by eutypa dieback. Australian and New Zealand Grapegrower and Winemaker **461a**, 76–77.

Creaser, M.L. and Wicks, T.J. (2002) Short–term effects of remedial surgery to restore productivity to eutypa dieback affected vines. Australian and New Zealand Grapegrower and Winemaker **461a**, 73–75.

Lardner, R., Mahoney, N., Molyneux, R. and Scott, E. (2002) Eutypa dieback: development of early diagnostic techniques. Australian and New Zealand Grapegrower and Winemaker **461a**, 78–79.

Eutypa dieback webpage at:
http://www.sardi.sa.gov.au/pages/hort/hort_crops/grapes/eutypa_trunk_disease/eutypapage1.htm

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.

Edwards J., Pascoe I.G., Joyce D.C., Cottral E.H., Taylor P.A., Lardner R., Scott E., Creaser M., Sosnowski M., Wicks T., Wiechel T. and Cole F.M. (2003) Managing grapevine trunk diseases (Petri disease, Esca, eutypa dieback and others) that threaten the sustainability of Australian viticulture. Interim Final Report to GWRDC, Project No. CRCV 2.2.1.

Sosnowski, M.R., Creaser, M.L. and Wicks, T.J. (2003) Managing eutypa dieback of grapevines: research update and future directions. The Australian and New Zealand Grapegrower and Winemaker **473a**, 88–89.

Lardner, R., Stummer, B. and Scott, E. (2004) Eutypa dieback: progress towards developing early diagnostic techniques. Australian Viticulture **8**, 51–53.

Sosnowski, M. (2004) Remedial Surgery for eutypa dieback – Research in McLaren Vale. The Grower's Choice (McLaren Vale Grape, Wine and Tourism), January 2004. p 4.

Sosnowski, M.R., Creaser, M.L. and Wicks, T.J. (2004). Evaluating fungicides as pruning wound treatments to control eutypa dieback. The Australian and New Zealand Grapegrower and Winemaker **485a**, 51–53.

Sosnowski, M.R., Creaser, M.L. and Wicks, T.J. (2004). Managing eutypa dieback of grapevines by remedial surgery. The Australian and New Zealand Grapegrower and Winemaker **488**, 35–39.

Sosnowski, M., Hall, B., Scott, E. and Wicks, T. (2004). International expert visits South Australia to update industry on the latest grapevine disease research in California. The Australian and New Zealand Grapegrower and Winemaker **485**, 20–24.

Sosnowski, M., Lamb, D., Gubler, D. and Rawnsley, B. (2004) Experts' insights into vine disease. Naracoorte Herald, February 12, 2004, p 2.

John, S., Scott, E., Wicks, T. and Hunt, J. (2005) Potential of Trichoderma harzianum and Fusarium lateritium to protect grapevines from eutypa dieback. The Australian and New Zealand Grapegrower and Winemaker, **497a**, 18–21

Lardner, R., Mahoney, N., Zanker, T., Molyneux, R. and Scott, E. (2005) Early detection of eutypa dieback using biochemical markers. The Australian and New Zealand Grapegrower and Winemaker **500**, 64–66

Sosnowski M. (2005). Eutypa dieback – an emerging issue in the Adelaide Hills. Adelaide Hills Wine Region Newsletter Summer 2005. p 6.

Sosnowski, M., Edwards, J., Lardner, R., Wicks T. and Scott, E. (2005) What's happening in the world of grapevine trunk diseases? The Australian and New Zealand Grapegrower and Winemaker **498**, 18–21.

Sosnowski, M., Lardner, R., Wicks, T. and Scott, E. (2005) A rapid method of screening grapevine cultivars for susceptibility to eutypa dieback. The Australian and New Zealand Grapegrower and Winemaker **493**, 14–16.

Sosnowski, M., Shtienberg, D., Creaser, M., Wicks T., Lardner, R and Scott, E. (2005) Unlocking the secrets of annual variation in eutypa dieback symptoms. The Australian and New Zealand Grapegrower and Winemaker **497a**, 7–12.

Sosnowski, M., Lardner, R., Wicks, T. and Scott, E. (2006) The spread of *Eutypa lata* within grapevines – implications for management of eutypa dieback. The Australian and New Zealand Grapegrower and Winemaker **509a,** 27-30.

Appendix 1.5. Presentations

Presentations relating to work on the project include those listed below. Oral and poster presentations on eutypa dieback management and diagnosis were made at Industry workshops, CRCV Conferences and Review Meetings and at international workshops and conferences.

International presentations

Creaser, M.J. (2000) Control of *Eutypa lata*. Paper presented at the 5th International Symposium on Cool Climate Viticulture and Oenology, Melbourne, 19 January.

Creaser, M.J. and Wicks, T.J. (2001) Yearly variation in symptom expression of eutypa dieback and its effect on grapevine yield. Paper presented at the 13th Biennial Australasian Plant Pathology Society Conference, Cairns, 27 Sept.

Creaser, M.J. and Wicks, T.J. (2001) Yearly variation in symptom expression of eutypa dieback and the relationship to grapevine yield. Paper presented at the 2nd International Council on Grapevine Trunk Diseases Workshop, Lisbon, Portugal, 14–15 Sept.

Creaser, M.J, Scott, E.S. and Wicks, T.J. (2001) Eutypa dieback in Australia: a new research initiative. Invited paper presented at the 7th Unified Wine and Grape Symposium, Sacramento, USA, 25 January.

Lardner, R. (2001) Early diagnosis of eutypa dieback. Paper presented at a meeting of the American Vineyard Foundation Project on eutypa dieback, Western Regional Research Center, Albany, California, 5 November.

Creaser, M.J. and Wicks, T.J. (2003) Short–term effects of remedial surgery to restore productivity to eutypa dieback affected vines. Paper presented at the 4th International Workshop for Grapevine Trunk Diseases, Christchurch, New Zealand, 1–2 February.

Lardner, R., Mahoney, N., Stummer, B., Molyneux, R., and Scott, E. (2003) Early diagnosis of eutypa dieback of grapevines. Poster presented at the 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2–7 February.

Lardner, R., Stummer, B. and Scott, E. (2003) Molecular and biochemical characterisation of *Eutypa lata*. Paper presented at the 4th International Workshop for Grapevine Trunk Diseases, Christchurch, New Zealand, 1–2 February.

Wiechel, T.J., Creaser, M.L., Cole, M.F. and Wicks, T.J. (2003) Secondary metabolites produced by southern Australian isolates of *Eutypa lata*. Poster presented at the 4th International Workshop for Grapevine Trunk Diseases, Christchurch, New Zealand, 1–2 February.

Wiechel, T.J., Creaser, M.L., Cole, M.F. and Wicks, T.J. (2003) Secondary metabolites produced by southern Australian isolates of Eutypa lata. Poster presented at the 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2–7 February.

John, S., Malone, J., Scott, E.S., Wicks, T.J. and Hunt, J.S. (2005) Biological control of eutypa dieback in Australia: field evaluation of fungal antagonists. 4th International Workshop on Grapevine Trunk Diseases, Stellenbosch, South Africa, 20–21 January 2005. p 84.

Lardner, R., Mahoney, N., Scott, E. and Molyneux, R. (2005) Biochemical detection of *Eutypa lata* in grapevines. 4th International Workshop on Grapevine Trunk Diseases, Stellenbosch, South Africa, 20–21 January 2005. p 35.

Lardner, R., Stummer, B. and Scott, E. (2005) Molecular diagnostic tools for the detection of *Eutypa lata* in grapevines. 4th International Workshop on Grapevine Trunk Diseases, Stellenbosch, South Africa, 20–21 January 2005. p 36.

Molyneux, R.J., Mahoney, N., Smith, L.R., Rolshausen, P.E., Lardner, R. and Scott, E. (2005) Diagnosis of *Eutypa* infection by metabolite analysis. 4th International Workshop on Grapevine Trunk Diseases, Stellenbosch, South Africa, 20–21 January 2005. p 18.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Effect of Brotomax™ applied to eutypa dieback–affected grapevines in South Australia. 4th International Workshop on Grapevine Trunk Diseases, Stellenbosch, South Africa, 20–21 January 2005. p 93.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Field evaluation of pruning wound treatments for control of eutypa dieback. 4th International Workshop on Grapevine Trunk Diseases, Stellenbosch, South Africa, 20–21 January 2005. p 85.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Field evaluation of pruning wound treatments for control of eutypa dieback. 43rd Southern African Society for Plant Pathology Congress, Hartenbos, South Africa, 23–26 January 2005. p 4.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Managing grapevines infected with eutypa dieback using remedial surgery. 4th International Workshop on Grapevine Trunk Diseases, Stellenbosch, South Africa, 20–21 January 2005. p 92.

Sosnowski, M.R., Creaser, M.L. and Wicks T.J. (2005) Managing grapevines infected with eutypa dieback using remedial surgery. 43rd Southern African Society for Plant Pathology Congress, Hartenbos, South Africa, 23–26 January 2005. p 89.

Gubler, W.D., Edwards, J., Sosnowski, M., Mugnai, L., Jaspers, M. and Mundy, D. (2006). Grapevine Trunk Diseases. 6th International Cool Climate Symposium for Viticulture & Oenology, Christchurch, New Zealand, 6–10 February 2006. p.170.

Sosnowski, M., Shtienberg, D., Creaser, M., Wicks, T., Lardner, R. and Scott, E. (2006) Climatic factors cause seasonal variation in the foliar symptoms of eutypa dieback. 5th International Workshop on Grapevine Trunk Diseases, Davis, USA, 11–12 Sept 2006. (in press)

Sosnowski, M., Wicks, T., Lardner, R. and Scott, E. (2006) Influence of cultivar and fungal isolate on the spread of Eutypa lata within grapevines. 5th International Workshop on Grapevine Trunk Diseases, Davis, USA, 11–12 Sept 2006. (in press)

National presentations

Creaser, M. (2000) IPM Viticulture: Research to Practice Training Workshop, McLaren Vale, 15 Nov 2000.

Creaser, M. (2000) Eden Valley Grapegrowers Association, 11 October 2000.

Creaser, M. (2000) CRC for Viticulture Program Two Meeting, Adelaide, 5 October 2000.

Creaser, M. (2000) NSW Wine Industry Association – Vine health for long term profitability, presentation and workshop, Wagga Wagga, NSW, 21 Jul 2000.

Creaser, M. (2000) Coonawarra Grapegrowers Association, several occasions in 2000.

Lardner, R. (2000) Early diagnosis of eutypa dieback in grapevines. Seminar presented to the Department of Applied Molecular Ecology, University of Adelaide, 23 October 2000.

Creaser, M.L. and Wicks, T.J. (2001) Yearly variation in eutypa dieback symptoms and the relationship to grapevine yield. In proceedings of the 13th Biennial Australasian Plant Pathology Society Conference, Cairns, 27 September 2001.

Creaser, M.L. and Wicks, T.J. (2001) 11th Australian Wine Industry Technical Conference – W35 Pest and disease identification and monitoring, Adelaide, 11 October 2001.

Creaser, M.L., Wicks, T.J., Lardner, R. and Scott, E.S. (2001) 11th Australian Wine Industry Technical Conference – W76 Grapevine trunk diseases, Adelaide, 8 October 2001.

Creaser, M.L., Wicks, T.J., Lardner, R., Scott, E.S., Stummer, B.E. and Cole, M.F. (2001) Eutypa dieback Project. CRC for Viticulture Program Two Second Year Review, Adelaide, 7–8 May 2001.

Creaser, M. (2002) IPM Viticulture: Research to Practice Training Workshop, Barossa Valley, 23 September 2002.

Creaser, M. (2002) Clare Valley Growers Meeting, Clare, 17 September 2002.

Creaser, M. (2002) Coonawarra Grapegrowers Vine Health Seminar, Coonawarra, 7 Jun 2002.

Creaser, M. (2002) CRC for Viticulture Program Review Meeting, Mildura, 19 Jun 2002.

Creaser, M. (2002) IHD Grapevine Conference, Tanunda, 19 August 2002.

Creaser, M. (2002) WestfarmersLandmark – IPM Viticulture: Research to Practice Training Workshop, McLaren Vale, 3 Jul 2002.

Lardner, R. (2002) Early diagnosis of eutypa dieback in grapevines. Seminar presented to the Department of Applied Molecular Ecology, University of Adelaide, 4 Apr 2002.

Lardner, R. (2002) Early diagnosis of eutypa dieback. Seminar presented at the CRCV annual symposium, 18 Jun 2002.

Lardner, R. (2002) eutypa dieback: An overview of the disease and current research. Seminar presented to the McLaren Vale Grapegrowers Association, 27 August 2002.

Lardner, R. (2002) Molecular and biochemical detection of eutypa dieback in grapevines. Seminar presented to the Department of Applied Molecular Ecology, University of Adelaide, 18 September.

Lardner, R. (2002) Molecular and biochemical diagnosis of eutypa dieback. Seminar presented to the Phylloxera and Grape Industry Board of South Australia, 19 August 2002.

Lardner, R. (2003) Early diagnosis and detection of eutypa dieback of grapevines. Seminar presented to the Discipline of Plant and Pest Science, University of Adelaide, 30 June 2003.

Scott, E.S., Lardner, R., Sosnowski, M.R. and Wicks, T.J. (2003) Diagnosis and management of eutypa dieback. CRCV Program 2 Year 5 Review, Adelaide, 4–5 September.

Scott, E.S., Lardner, R., Sosnowski, M.R., Wicks, T.J., Wiechel, T. and Cole, F.M. (2003) Managing grapevine trunk diseases: eutypa dieback. CRCV Program 2 Year 5 Review, Adelaide, 4–5 September.

Sosnowski, M. (2003) Hunter Valley Vineyard Seminar – Trunk Disease Management "Understanding the Big Picture" Rosemount Estate, Denman, 28 May 2003.

Sosnowski, M. (2003) Mudgee Vineyard Seminar – Trunk Disease Management "Understanding the Big Picture" The Australian Rural Education Centre, Mudgee, 30 May 2003.

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Appendix 1.6. Refereed scientific journal articles published during the project

Secondary metabolite production by the fungal pathogen *Eutypa lata:* Analysis of extracts from grapevine cultures and detection of those metabolites *in planta*

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Abstract

Eutypa dieback of grapevines is caused by the fungal pathogen *Eutypa lata* and reduces vineyard longevity worldwide. Early detection could reduce losses due to this disease, so our aim was to identify acetylenic phenol metabolites of *E. lata* that could prove suitable as chemical markers in an early diagnostic test for the pathogen. Accordingly, secondary metabolite production by 30 isolates of *E. lata* grown on media derived from canes of three grapevine cultivars was analysed using HPLC. Six metabolites, namely eutypinol, methyl eutypinol, eulatachromene, eutypine, 2-*iso*-propenyl-5-formylbenzofuran and eulatinol, were detected in culture filtrates. Most abundant were eutypinol and methyl eutypinol, produced by 97 and 83% of isolates, respectively. There was no apparent correlation between secondary metabolite production on media containing milled canes from the three cultivars of grapevine, and the field tolerance of these same cultivars to Eutypa dieback. When various other fungi commonly isolated from grapevine trunks in Australia were grown on milled cane, no secondary metabolites characteristic of *E. lata* were detected, suggesting such compounds are specific to *E. lata*. To examine the detection of secondary metabolites *in planta*, micropropagated grapevine plantlets were treated with purified or crude culture filtrates from nine isolates of *E. lata* grown on malt yeast broth. Various secondary metabolites were identified in treated plantlets, however, no single compound was detected consistently. Eutypinol was detected in micropropagated grapevine plantlets inoculated with mycelium of *E. lata*, however, no metabolites were detected in the sap of vines which had been artificially inoculated with the pathogen.

Introduction

Eutypa dieback has a significant economic impact in vineyards worldwide. In some Australian vineyards more than 60% of vines show foliar symptoms of the disease (Wicks and Hall 1997), and up to 100% may exhibit the characteristic necrosis of woody tissue caused by *Eutypa lata* (Creaser and Wicks 2002). Studies in California showed that over 90% of vines may be affected by the time vineyards reach 20 years of age (Duthie et al. 1991), with older vineyards showing yield decreases of up to 83% (Munkvold et al. 1994). Ultimately, infected vines will die.

Shoot symptoms include dwarfing of internodes, leaf tattering and foliar necrosis. These symptoms are clearly visible in spring, although they quickly become obscured by vigorous growth from neighbouring healthy vines and may be confused with other diseases or disorders. There is considerable annual variation in symptom expression, with vines commonly displaying symptoms one year but not in the subsequent year (Creaser and Wicks 2001). Foliar symptoms have been attributed to toxins produced by *E. lata* in the vascular tissue of the wood. *E. lata* colonises vines through fresh wounds in mature wood and grows slowly throughout the vascular tissue. Foliar symptoms may not become evident until 3–8 years after infection (Carter 1991, Moller and Kasimatis 1978). Hence, by the time foliar symptoms are visible, the pathogen may have spread extensively throughout the vine.

DNA-based markers to identify *E. lata* within infected woody tissues are available (Lardner et al. 2005, Lecomté et al. 2000). However, this is a destructive assay. Furthermore, although DNA-based identification is a sensitive technique, this method relies on sampling host tissue that contains mycelium of *E. lata*, so that false negative results are possible if samples are taken from healthy tissue within an infected vine. In contrast, metabolites of *E. lata* are likely to be distributed throughout the vascular tissue and foliage of infected vines, especially in spring when

foliar symptoms are most evident. Hence, diagnosis based on identifying specific metabolites of *E. lata in planta* may enable early and more reliable detection of the pathogen before it spreads extensively throughout the vine.

The phytotoxin eutypine was originally implicated as the compound responsible for the foliar symptoms of Eutypa dieback (Tey Rulh et al. 1991). However, we previously found that eutypine is not produced by all isolates of *E. lata*, and that it readily undergoes facile cyclisation to a benzofuran compound (Molyneux et al. 2002). Furthermore, *E. lata* produced a range of other metabolites in artificial culture. Previous studies have shown that these metabolites have varying degrees of phytotoxicity, with some compounds, for example eutypinol and siccayne, showing no phytotoxicity when applied to grapevine leaf discs and others, including eutypine and eulatachromene, causing widespread necrosis of leaf discs (Mahoney et al. 2003, Smith et al. 2003). We also found that the amount and type of metabolite produced varied according to the fungal isolate and growth medium (Mahoney et al. 2003, Mahoney et al. 2005). Originally, we hoped that a chemical marker could be detected remote from the site of infection, in the sap or foliage of infected vines. We anticipated that low levels of metabolites of *E. lata* would be present before foliar symptoms became visible, and that this could be used as an early diagnostic tool for the pathogen. However, working *in vivo*, metabolites of *E. lata* have so far only been found within the woody tissues of infected vines.

Those metabolites could nevertheless prove diagnostic, and here we report on a series of experiments conducted to clarify what metabolites were consistently produced by *E. lata* in artificial culture and to obtain information on detecting these metabolites *in planta*. Because *E. lata* is an out-crossing species that displays considerable genetic and pathogenic variation (Péros and Berger 2003, Sosnowski et al. 2006), we analysed 30 isolates to obtain a representative picture of secondary metabolite production by the pathogen.

Firstly, we analysed secondary metabolite production by 30 isolates of *E. lata* which were grown on milled cane from three cultivars of grapevine. Our goals were (1) to identify a chemical marker that was produced by the majority of isolates of *E. lata* and (2) to determine whether cultivar tolerance to Eutypa dieback influenced the amount or type of secondary metabolite produced. We also analysed secondary metabolite production by 18 isolates of other grapevine-inhabiting fungi after growth on milled grapevine cane to confirm that the metabolites detected here were specific to *E. lata*.

Secondly, we grew nine isolates of *E. lata* on malt yeast broth, previously shown to be conducive to the production of a range of metabolites by *E. lata*. Micropropagated grapevine plantlets were then treated with purified or crude filtrates taken from these cultures. Our aim was to see whether metabolites of *E. lata* could be detected in grapevine tissue, and to gather information about the fate of such metabolites *in planta*.

Thirdly, to determine whether chemical markers indicative of *E. lata* could be detected in infected vines, we used HPLC to identify such metabolites in both micropropagated and potted vines which had been previously inoculated with mycelium from *E. lata*.

Methods

Isolates and culturing

Isolates of *E. lata* (Table 1) were obtained from perithecia on mature wood or from hyphae in infected cordons, as described by Carter (1991). Cultures were maintained in the dark on potato dextrose agar (PDA, Difco) at 25°C and stored in sterile distilled water at 4°C. For secondary metabolite analysis, media were derived from 1-year-old dormant canes of cultivars Merlot, Semillon and Shiraz, which were ground in a Wiley mill with a 1 mm screen. Each isolate was cultured in a 250 mL flask containing 10 g ground cane, 1 g sucrose and 50 mL reverse osmosis water that was sterilised by autoclaving. Flasks were inoculated with 5–8 mycelium plugs (approximately 2 square millimetres), taken from the margin of actively growing colonies on PDA. Cultures were maintained in the dark at 25°C for 20 d. Species other than *E. lata* were cultured on Shiraz medium only. These species included five isolates of *Phaeomoniella chlamydospora*, three isolates each of *Botryosphaeria* sp. and *Fomitiporia* sp., two isolates each of *Cryptovalsa ampelina* and *Phaeoacremonium aleophilum* and one isolate each of *Fomitiporia australiensis*, *Botryosphaeria ribis* and *Libertella* sp.

Isolation and analysis of secondary metabolites specific to E. lata

After incubation for 20 d, cultures were mixed with 100 mL double distilled (dd) water and mycelium was disrupted using a spatula. The resulting suspension was sonicated for 15–30 s then filtered through Whatman No. 4 filter paper. The filtrate was partitioned with an equal volume of chloroform and the aqueous phase discarded. The organic phase was partitioned with an equal volume of water, the aqueous phase discarded and chloroform was removed under reduced pressure. The residue was re-suspended in 10 mL acetonitrile, and liquid removed under reduced pressure. The residue was dissolved in methanol (1 mL) and filtered through a 0.45 µm 3 mm syringe filter (Gelman). Samples (20 µL) were analysed by HPLC using a Microsorb C_{18} 5 µm column, 250 × 4.6 mm i.d. (Varian) with a gradient elution at 1 mL/min of 100% water with 0.5% acetic acid to 100% acetonitrile over 30 min and held at 100% acetonitrile for 5 min. Photodiodearray detection (Agilent 1100 DAD) was used to identify metabolites. Amounts of previously characterised compounds were determined by reference to standard curves prepared for each compound. Reference standards were synthesised as described by Smith et al. (2003) and were linear over the range tested, 0.2–20 µg/20 µL injection. Amounts of any previously unidentified metabolites of *E. lata* were not quantified, but were instead represented by either '+' for presence or '–' for absence.

Detection of secondary metabolites in micropropagated vines treated with metabolites of E. lata

To see whether secondary metabolites of *E. lata* could be detected *in planta*, micropropagated grapevine shoots and

Table 1. Summary of secondary metabolite production by 30 isolates of *E. lata* on milled cane from three grapevine cultivars.

Isolate	Host species & origin	Metabolites (µg)														
		Eutypinol			Methyl eutypinol			Eulatachromene			Eutypine			Benzofuran[1]		
		Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon	Shiraz	Merlot	Semillon
01017B	*Vitis vinifera*, NSW	63	19	13	3.3	1	1.2	—	—	—	1.1	—	—	—	0.4	0.1
1776	*V. vinifera*, SA	2.5	—	1.5	—	—	—	—	—	—	—	—	—	—	—	—
200/2	*V. vinifera*, SA	351	440	586	23	5.2	13	26	28	21	2.9	2.4	—	3	—	0.9
200/3	*V. vinifera*, SA	466	111	615	43	9.4	31	25	—	23	16	—	19	1	6.1	1.1
83330	*Prunus persica*, SA	1.1	1.8	96	—	—	8.8	—	—	10	—	—	2.6	—	—	1.1
83339	*Quercus* sp.	661	236	1380	126	7.4	138	28	5.5	36	—	—	—	—	0.5	—
Bx 1-10	*V. vinifera*, France	197	35	282	59	2	24	—	—	4.9	—	—	5.1	3.5	2.4	1.5
E1	*V. vinifera*, SA	295	56	81	47	5.2	3.8	—	—	—	12	—	0.7	10	0.7	0.6
E120	*V. vinifera*, California	172	88	180	—	—	—	5.3	5.3	8.8	4.2	0.7	13	0.3	1.9	3
E125	*V. vinifera*, California	869	255	547	47	1.7	14	24	11	17	57	1.8	—	75	1.9	16
EL3	*V. vinifera*, S. Africa	387	266	130	53	11	15	28	11	3.8	—	1.5	—	1	0.6	0.4
EL4	*V. vinifera*, S. Africa	114	279	103	28	10	4.9	12	21	5.6	27	9.1	1.1	1.2	3.1	0.9
HT01	*Prunus armeniaca*, SA	491	105	417	59	4.8	15	20	1.5	9.3	7.6	2	4.1	2.1	1.4	1.3
JB16	*V. vinifera*, SA	706	486	701	164	43	84	33	16	47	—	19	—	—	12	—
M266	*V. vinifera*, France	1070	13	137	—	—	1.3	—	—	1.3	18	—	—	—	0.1	1.9
M279	*Prunus armeniaca*, Vic	—	7.8	20	7.0	6.2	27	—	1	—	—	—	7.3	—	2.7	27
M280	*V. vinifera*, Vic	179	522	491	11	8.5	—	0.8	6.9	7.3	2.7	4	18	12	11	—
M295	*V. vinifera*, Vic	—	—	1.4	—	—	—	—	—	—	—	—	—	—	—	—
M302	*V. vinifera*, Vic	131	41	75	9.6	1.5	2.6	4.2	—	1.8	12	—	1.2	1.2	0.4	0.1
M335	*V. vinifera*, Vic	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
NO3	*V. vinifera*, SA	303	19	82	51	0.9	3.9	—	—	—	—	—	—	10	0.4	4.2
NO4	*V. vinifera*, SA	610	199	487	96	21	55	13	4.5	11	18	—	—	6	3.6	12
NO7	*V. vinifera*, SA	216	224	310	259	36	85	9.7	8.7	7.8	—	—	—	10	0.3	1
NO8	*V. vinifera*, SA	421	39	376	23	1.6	9.2	12	—	8	9.4	—	—	2.1	0.5	9
P#1 SS12	*Pyrus communis*, SA	—	25	19	—	—	—	—	—	—	—	—	—	—	0.4	0.1
RB440	*V. vinifera*, France	24	203	126	7.2	18	21	—	4.7	3.5	7.2	4.2	12	2	2.2	3.6
SS2	*V. vinifera*, SA	—	—	48	—	—	4.5	—	—	1.6	—	10	1.3	—	—	37
SS6	*Viburnum opulus*, SA	3.8	16	18	6.1	2.3	7.3	—	—	—	1.4	1	3.1	2.7	6.3	9
SS10	*V. vinifera*, SA	3.8	44	17	0.3	2	1	—	—	—	0.05	—	—	—	0.3	0.1
SS11	*V. vinifera*, SA	2	12	9.3	0.5	1.3	1.2	—	—	—	—	0.2	0.3	0.2	—	—
	Total	7738	3745	7350	1124	199	572	241	125	229	196	56	89	144	59	132

— = not detected

[1] 2-*iso*-propenyl-5-formylbenzofuran

Detection limits (µg/mL) at 254 nm:

Eutypinol	0.03	Benzofuran	0.005
Methyl eutypinol	0.03	Eulatinol	0.01
Eulatachromene	0.05	Siccayne	0.01
Eutypine	0.01		

plantlets (cultivar Cabernet Sauvignon) were treated with purified or crude culture filtrates from nine isolates of *E. lata* (isolates E1, E120, E125, M266, M279, M280, M335, NO4 and SS6) grown on malt yeast broth (MYB; 10 g/L malt extract, 20 g/L yeast extract, Difco). MYB was selected for use in this experiment as we have previously shown that *E. lata* produces a wide range of secondary metabolites on this medium (Mahoney et al. 2003).

Conical flasks (250 mL) containing 50 mL MYB were inoculated with *E. lata* as above. Cultures were maintained for 30 d at 25°C in the dark. Three cultures were initiated for each isolate. After 30 d, replicate cultures were pooled, then sonicated (15–30 s) and filtered through Whatman No. 4 filter paper. The filtrate was divided into three equal portions. Two portions were extracted to provide purified filtrates, as described above, except that di-ethyl ether was used as the solvent rather than chloroform. The first purified filtrate was re-suspended in methanol and analysed using HPLC as described above. This provided a reference sample of known composition for each of the nine isolates.

The second purified filtrate from each isolate was re-suspended in ethylene glycol (200 µL) then added to 5 mL sterile dd water. Shoots (four internodes and the apex) were excised from micropropagated grapevine plantlets, placed in the resulting 5.2 mL of purified filtrate and incubated at 25°C (16 h light, 8 h dark) in 120 mL polypropylene tissue culture vessels for 10 d. After 10 d, shoots were rinsed in dd water to remove any filtrate, and dried on paper towel. Shoots were then ground in liquid nitrogen, mixed in 20 mL dd water, sonicated for 60 s and filtered through Whatman No. 4 filter paper. The filtrate was partitioned with an equal volume of di-ethyl ether, and

metabolites extracted and analysed by HPLC as described above. Following the removal of excised shoots, metabolites were also extracted from the ethylene glycol/water solution by making the volume up to 20 mL with dd water, filtering through Whatman No. 4 filter paper, then extracting with di-ethyl ether and subjecting to HPLC as described above.

The third portion of each MYB culture was sterilised by passing through a 0.2 µm filter, and the resulting crude filtrate (50 mL) was applied to the roots of micropropagated grapevine plantlets established on filter paper bridges in half-strength liquid MS medium (Murashige and Skoog 1962) in 250 mL polypropylene tissue culture vessels. The MS medium was removed before the addition of the crude filtrate. Plantlets were exposed to crude filtrates for 10 d, then cut off at root level and rinsed in dd water. Shoots were ground in liquid nitrogen, sonicated and extracted with di-ethyl ether as described above. Metabolites were also extracted from the remaining material (a mixture of plantlet roots and crude filtrate) by grinding in liquid nitrogen and extracting with an equal volume of di-ethyl ether. The extracts were then analysed by HPLC as above. Control plants, treated with solvent or MYB only, were included for each treatment.

Detection of secondary metabolites in micropropagated vines inoculated with mycelium of E. lata

Micropropagated grapevine plantlets (cultivar Cabernet Sauvignon) were inoculated with mycelium from five of the nine isolates of *E. lata* used above (isolates E1, E120, E125, M279 and M280). Six plantlets were inoculated for each isolate, and six uninoculated control plantlets were included. Plantlets were maintained on half-strength MS medium at 25°C (16 h light, 8 h dark) and were inoculated by placing slivers of mycelium taken from the margins of actively growing cultures on fresh wounds made on the stem of plantlets. Mycelium was held in place by applying a small amount of molten water agar immediately after inoculation. Plantlets were assessed for foliar symptoms 42 days after inoculation and metabolites were extracted and analysed using HPLC as described above.

Detection of secondary metabolites in infected vines

Metabolites were also extracted from the sap of potted vines which had been artificially inoculated with mycelium of ten isolates, individually, of *E. lata* in the shadehouse (Sosnowski et al. 2005a). For each isolate, two vines were selected, one which displayed intense foliar symptoms of Eutypa dieback within 8 months of inoculation, and one which had been inoculated with the same isolate but was asymptomatic. Sap was extracted from each vine 20 months after inoculation using a pressure chamber. This was achieved by placing the entire vine in the chamber, which was pressurised until sap began exuding from a freshly cut petiole. A pressure chamber was used on the assumption that metabolites of *E. lata* are present in the xylem sap of infected vines, which is exuded when vines are placed under pressure. For each vine, sap was collected over a four-hour period. Sap was also extracted from ten control vines, five of which were inoculated with blank agar plugs and five of which were uninoculated. Metabolites were extracted from the sap using di-ethyl ether and analysed using HPLC as described above.

Results

Isolation and analysis of secondary metabolites specific to E. lata

Five phenolic metabolites, namely eutypinol, methyl eutypinol, eulatachromene, eutypine and its benzofuran derivative, 2-*iso*-propenyl-5-formylbenzofuran, were commonly detected in extracts from cultures of *E. lata* grown for 20 d on milled wood of each cultivar. A quinol compound, eulatinol, was also produced by some isolates. Secondary metabolite production by all isolates on each cultivar is summarised in Table 1. Four of the 30 isolates produced all six metabolites, 14 isolates produced five metabolites and seven produced four metabolites. One isolate, M335, produced no secondary metabolites that were detectable by HPLC; the remaining isolates of *E. lata* produced between one and three metabolites. The other species tested did not produce any detectable acetylenic phenol metabolites.

The most frequently produced and abundant compound was eutypinol, produced by 29 of the 30 isolates (all except M335). Each of the 29 isolates produced eutypinol when grown on Semillon. Three isolates (1776, M295 and SS2) did not produce eutypinol on Merlot, and four (M279, M295, P#1SS12 and SS2) did not produce eutypinol on Shiraz. Amounts of eutypinol varied depending on fungal isolate and cultivar. For example, isolate M266 produced 1070, 137 and 13 µg of eutypinol following growth on Shiraz, Semillon and Merlot, respectively. However, there was no correlation between amount of eutypinol and cultivar; whereas isolate M266 produced most eutypinol on Shiraz, isolates 83339 and RB440 produced maximum amounts on Semillon and Merlot, respectively (Table 1). The amount of eutypinol produced ranged from 1 µg (isolate 83330, Shiraz) to 1380 µg (83339, Semillon). Eutypinol comprised 82% of the total metabolites produced when isolates were grown on Shiraz, 88% on Semillon and 90% on Merlot. Methyl eutypinol was also relatively abundant, produced by 25 isolates in amounts ranging from 0.3 µg (SS6, Shiraz) to 259 µg (N07, Shiraz). Taken together, eutypinol and methyl eutypinol comprised approximately 94% of the total acetylenic phenol metabolites produced on each cultivar.

Eutypine, present in smaller quantities than eutypinol or methyl eutypinol, was produced by 23 isolates, in amounts ranging from 0.05 µg (SS10, Shiraz) to 57 µg (E125, Shiraz). Six isolates produced eutypine on milled wood of all three cultivars, seven on two cultivars and ten isolates on a single cultivar. The benzofuran compound, eulatachromene and eulatinol were produced by 27, 20 and 5 isolates, respectively (Table 1).

There was no apparent correlation between cultivar and type of secondary metabolites produced. On each cultivar, eutypinol was predominant, followed by methyl eutypinol and eulatachromene. The benzofuran compound was the next most predominant metabolite on Semillon and Merlot, whereas on Shiraz slightly more

eutypine was produced than benzofuran. Eulatinol, not detected in extracts from isolates grown on Merlot, was produced in the smallest amounts, by five isolates. Total metabolite production was considerably greater on Shiraz and Semillon (9457 and 8379 µg, respectively) than on Merlot (4184 µg).

Detection of secondary metabolites in micropropagated vines treated with metabolites of E. lata

Following growth of nine isolates of *E. lata* on MYB, the six compounds identified above, and two other compounds, methyl eutypine and siccayne (Deswarte et al. 1996, Molyneux et al. 2002), were detected in purified culture filtrates analysed by HPLC. Eutypinol was the predominant compound, but was not produced by every isolate. Control shoots treated with ethylene glycol did not display any symptoms following 10 d of exposure. Excised shoots treated with metabolites from isolates E1 and M266 displayed bleaching of stems and necrosis of foliage after 10 d; shoots treated with metabolites from the remaining isolates were asymptomatic. All plantlets treated with crude MYB filtrate displayed widespread necrosis; this may be due to the pH of the medium, which becomes alkaline following incubation of *E. lata* (N. Mahoney, unpublished data).

Detection of metabolites of *E. lata* in treated plant material was somewhat inconsistent, with yields from treated plant material generally small relative to their amount in the purified culture filtrate. Analysis of excised shoots treated with purified culture filtrates showed that eutypinol could be detected in these shoots, although always in small quantities, and only when shoots were treated with extracts from isolates that produced more than 100 µg of eutypinol as detected in the purified culture filtrate analysed by HPLC. Other compounds, including methyl eutypinol, eulatachromene, benzofuran and eulatinol were also detected in excised shoots exposed to purified culture filtrates. Although eutypine was present in the filtrates of some isolates, it was never detected in treated plant material. The amount of metabolites recovered was generally less in rooted plantlets treated with crude filtrates than in excised shoots treated with purified filtrates. Metabolites detected in plant material treated with filtrates from representative isolates are shown in Table 2.

Two novel compounds, with retention times of 16.5 and 19.5 min, were also detected. These compounds, which have not been fully characterised, were commonly present at higher levels in treated shoots and plantlets than in the purified culture filtrate analysed by HPLC; the compound with a retention time of 19.5 min was not present in purified culture filtrates, but was detected in excised shoots treated with purified filtrates and in plantlets treated with crude filtrates, as well as in the reservoir of ethylene glycol/filtrate solutions remaining after exposure to excised shoots (Table 2).

Detection of secondary metabolites in micropropagated vines inoculated with mycelium of E. lata

Forty-two days after inoculation, some, but not all plantlets displayed foliar symptoms similar to those seen in vines naturally infected with *E. lata*. HPLC analysis identified eutypinol in plantlets inoculated with mycelium from four isolates (all except E125), however, no other metabolites characteristic of *E. lata* were detected. Eutypinol was detected in symptomatic and asymptomatic plantlets, but never in more than two out of six replicates.

Detection of secondary metabolites in infected vines

HPLC analysis of sap taken from potted vines which had been inoculated with *E. lata* did not identify any characteristic metabolites in symptomatic or asymptomatic vines. The volume of sap exuded from each vine over a four-hour period varied from approximately 1 to 26 mL and was generally considerably less in infected vines, presumably because the xylem tissue had become blocked following infection by *E. lata*.

Discussion

Our present study provides the first report of analysis of secondary metabolites from a wide range of isolates of *E. lata* grown on milled cane from various grapevine cultivars. The study supports the suggestion by Mahoney et al. (2003) that eutypinol is suited for use in an early diagnostic test based on secondary metabolite production *in vitro*. However, methyl eutypinol and the benzofuran compound, which were produced by 83 and 90% of isolates respectively, may also be suitable chemical markers.

Eutypinol is not phytotoxic when applied to grapevine leaf discs (Mahoney et al. 2003, Smith et al. 2003). However, this absence of phytotoxicity does not preclude using eutypinol as a chemical marker. Rather, pathogenicity of *E. lata* is not solely a consequence of the production of phenolic metabolites, but reflects the ability of the pathogen to colonise grapevine wood and degrade the xylem tissue. Hence, the presence of *E. lata* in vines as illustrated by a non-toxic metabolite such as eutypinol is sufficient indication of infection. Based on clinical evidence, the pathogen will eventually colonise the entire trunk or cordon, either killing the entire vine or a significant portion of it, regardless of whether foliar symptoms are expressed.

Eutypinol was produced by 29 isolates, however not every isolate produced eutypinol on every cultivar. Similar patterns of production were seen for other compounds. This is consistent with studies showing that production of certain metabolites by *Fusarium* spp. appears to be species-, isolate- and substrate-specific (Doohan et al. 2003). Factors that influence fungal secondary metabolite production remain unclear (Betina 1989, Kokkonen et al. 2005), with different compounds commonly produced on different substrates. Indeed, for *E. lata*, eulatinol was produced almost exclusively on artificial media, in particular on MYB, whereas other compounds, for example eutypine and eulatachromene, were more abundant when cultured on grapevine extracts (Mahoney et al. 2003).

Incubating the pathogen on cane extracts rather than artificial media was expected to provide a more accurate indication of metabolite production *in planta*. However, this technique would not provide information on plant-

Table 2. Detection of secondary metabolites in plantlets exposed to purified and crude culture filtrates from representative isolates of *E. lata* for 10 days

Isolate	Treatment	Metabolites (µg)									
		Eutypinol	Methyl eutypinol	Eulata-chromene	Eutypine	Methyl eutypine	Benzo-furan	Eulatinol	Siccayne	rt 16.5 min[1]	rt 19.5 min[1]
E125	Purified filtrate (HPLC)	203	4.1	7.8	6.1	3.5	10	21	—	+	-
	Excised shoot treated with purified filtrate	0.8	0.7	—	—	—	—	—	—	+	+
	Ethylene glycol solution	93	22	2.7	—	—	—	—	—	+	+
	Rooted plantlet treated with crude filtrate	—	—	—	—	—	—	—	—	+	-
	Crude filtrate/roots	3.7	12	—	—	—	—	35	—	+	+
M266	Purified filtrate (HPLC)	1273	—	58	21	—	3.6	—	—	-	-
	Excised shoot treated with purified filtrate	10	0.3	5.9	—	—	0.4	—	—	-	-
	Ethylene glycol solution	409	—	31	13	—	2.8	—	—	+	-
	Rooted plantlet treated with crude filtrate	0.2	—	—	—	—	—	—	—	+	-
	Crude filtrate/roots	36	—	—	12	—	5.6	—	—	+	+
N04	Purified filtrate (HPLC)	269	149	7	—	1.6	2.7	78	—	+	-
	Excised shoot treated with purified filtrate	2.4	2.7	—	—	—	0.1	1.3	—	-	+
	Ethylene glycol solution	148	81	6.3	—	1	5.5	45	—	+	-
	Rooted plantlet treated with crude filtrate	0.5	0.6	—	—	—	0.3	1.1	—	+	+
	Crude filtrate/roots	26	18	—	19	1.6	3.6	59	—	+	+

— = not detected

[1] Quantitative data for metabolites with retention times of 16.5 and 19.5 min could not be determined as these compounds have not been fully characterised. Presence of these compounds is therefore indicated by '+', and absence by '-'.

fungal interactions. For example, enzymatic detoxification of eutypine to eutypinol in living grapevine tissue, as reported by Colrat et al. (1999), would not occur on autoclaved cane. Likewise, the age of the cane may influence the metabolism of the pathogen – an outcome previously reported by Ferreira (1999) and Schmidt et al. (1999), who observed differences in growth rates and production of extracellular hydrolytic enzymes when growing *E. lata* on annual cane versus mature wood, respectively. Hence, the age of the wood on which *E. lata* is grown may also influence production of acetylenic phenol secondary metabolites.

Two factors known to influence secondary metabolite production are temperature and water status (Betina 1989, Kokkonen et al. 2005); this has been illustrated for various fungi, including *Phoma* sp. and *Fusarium* sp. (Baxter et al. 1998, Doohan et al. 2003). These factors have not been examined in relation to secondary metabolite production by *E. lata*. However, recent research (Sosnowski et al. 2005b) showed that high rainfall in October (spring) may be related to reduced severity of foliar symptoms of Eutypa dieback in some South Australian vineyards. Conversely, extremely low rainfall in spring may also be related to a decrease in the severity of foliar symptoms (Sosnowski et al. 2005b). It is possible that certain toxic secondary metabolites of *E. lata* are synthesised only in particular conditions. If metabolite production is strongly influenced by temperature, water availability or other environmental factors, this may help explain the variation in severity of foliar symptoms of Eutypa dieback observed from year to year (Creaser and Wicks 2001). Vineyard management practices, such as regulated deficit irrigation (Goodwin and Jerie 1992), could also influence the expression of foliar symptoms. Further research is clearly required to elucidate the complex interactions between the host, pathogen and environment on fungal establishment on host vines and progress of this disease.

Isolate M335 was confirmed as *E. lata* using DNA markers specific to the pathogen (Lardner et al. 2005). However, this isolate did not produce any secondary metabolites detectable by HPLC. Santos et al. (2002) reported inconsistent production of certain secondary metabolites following storage of isolates of *Penicillium expansum*. Conceivably, the response of fungi to preservation may be isolate-specific (Ryan et al. 2001, Santos et al. 2002), so it is possible that isolate M335, obtained from perithecia on dead grapevine wood, may have lost the ability to produce secondary metabolites following storage.

In this study, two cultivars that are tolerant to the effects of Eutypa dieback (Merlot and Semillon) and one susceptible cultivar (Shiraz) were selected to assess the influence of cultivar on secondary metabolite production. Although the metabolite profiles of individual isolates varied considerably, the general pattern of metabolite production was similar among cultivars, with eutypinol predominant. The total amount of secondary metabolites produced on Merlot was less than half that on Shiraz, however, a similar total amount of metabolites was produced on Semillon and Shiraz. In this respect then, there was no apparent correlation between amount or type of secondary metabolites produced, and tolerance of the grapevine cultivar to Eutypa dieback.

Analysis of micropropagated plantlets treated with cul-

ture filtrates showed low yields of metabolites from plant material. Although eutypinol was detected in excised shoots treated with purified culture filtrates, it was detected less consistently in intact plantlets treated with crude culture filtrates. This may be due to poor uptake of compounds by rooted plantlets, however, other compounds, in particular eulatinol, could be detected in rooted plantlets as well as in excised shoots. This implies that failure to detect some compounds is more likely caused by other factors, such as degradation of compounds when in aqueous solution, or perhaps by *in planta* conversion to compounds not detected using HPLC.

The detection of previously unidentified metabolites in treated plantlets, which were absent or present in very small amounts in purified culture filtrates, provides some evidence for the transformation of metabolites *in planta*. Time course experiments, in which metabolites are extracted from plantlets treated with identical culture filtrates after various periods of exposure, may provide more information on detecting these compounds *in planta*. Likewise, exposing plantlets to individual metabolites would provide information on the uptake and possible degradation of these compounds. Such transformed metabolites may prove to be suited for use in a rapid diagnostic test if they can be shown to be a reliable indicator of infection in diseased vines. Because other compounds such as methyl eutypinol and the benzofuran compound could be detected in treated plantlets, even when present in smaller amounts than eutypinol in the purified culture filtrates analysed by HPLC, the possibility of using these compounds as chemical markers may warrant further investigation.

HPLC analysis of plantlets inoculated with mycelium of *E. lata* showed that eutypinol could be detected in plant material infected with the pathogen. The detection of eutypinol in asymptomatic plantlets supported the hypothesis that metabolites of *E. lata* may be present in infected vines before foliar symptoms become visible. However, neither eutypinol nor any other metabolites were detected consistently in symptomatic plantlets inoculated with the pathogen. Perhaps the non-lignified tissue of micropropagated plantlets is not an appropriate substrate for the production of secondary metabolites by *E. lata*.

Our inability to detect any metabolites in the sap of symptomatic vines inoculated with characterised isolates of *E. lata* implies that these metabolites may not be suitable chemical markers for diagnosing Eutypa dieback. The observation by Mahoney et al. (2005) that metabolites of *E. lata* could not be detected in naturally infected vines showing foliar symptoms of Eutypa dieback also indicates that these metabolites may not be suitable chemical markers. Reasons for this remain unclear; it is possible that, following their entry into grapevine tissue, such metabolites are rapidly broken down into compounds that cannot be detected using HPLC. Alternatively, the suggestion of Mahoney et al. (2005) that such metabolites are not, in fact, translocated from the site of infection may also explain our inability to detect these compounds in the sap or foliage. Octave et al. (2006) isolated polypeptide compounds from a single isolate of *E. lata* grown in artificial culture and showed that when leaves were treated with such compounds, mesophyll cells underwent modifications similar to those observed in naturally infected vines. Hence, it is possible that such compounds may prove a more reliable indicator of infection by *E. lata*. However, before such research is initiated, it would be necessary to confirm that these polypeptides are produced by the majority of isolates of *E. lata*, and that they are not an artefact of the medium upon which the fungus is grown.

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Molecular identification and detection of *Eutypa lata* in grapevine

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Eutypa lata, the causal agent of Eutypa dieback of grapevines, is difficult to identify on the basis of colony morphology and is often out-competed by other fungi when isolated from wood. To facilitate diagnosis of the pathogen, we designed SCAR primers capable of amplifying DNA of *E. lata* and constructed a genomic DNA library from which DNA sequences specific to *E. lata* were identified and sequenced. SCAR primers were used to identify *E. lata* directly from culture without the requirement for DNA extraction or prolonged incubation periods and could also detect the pathogen in DNA isolated from grapevine wood. RFLP probes were used in slot-blot assays to detect the pathogen in DNA isolated from 1 yr old cane as well as from mature grapevine trunks. The markers developed in this study have the potential to be used as a research tool to gather information on the epidemiology of the disease and to assess the efficacy of potential control agents against *E. lata*.

INTRODUCTION

Eutypa lata, a major pathogen of grapevine (*Vitis vinifera*) throughout the world, causes Eutypa dieback, which affects mainly vines over 8 yrs old resulting in a gradual decline of health and yield. Premium red cultivars, for example, Cabernet Sauvignon and Shiraz, appear to be the most severely affected and in some Australian vineyards more than 60% of vines show symptoms of Eutypa dieback (Wicks & Hall 1997). In California over 90% of vines may be affected by the time vineyards reach 20 yr (Duthie *et al.* 1991), with yield decreases of up to 83% in older vineyards (Munkvold, Duthie & Marois 1994).

Vines become infected following germination of ascospores of *E. lata* in xylem vessels of fresh pruning wounds, then there is a latent period of 3–8 yr whilst the mycelium colonises the vascular tissue (Moller & Kasimatis 1978, Carter 1991). Cankers form around pruning wounds in older wood (English & Davis 1965) and necrosis of vascular tissue impedes water and nutrient movement within the plant. Cankers are difficult to detect because they may be covered with bark, but a characteristic wedge-shaped area of discoloured wood can be seen in cross-sections of the trunk. If the disease progresses unchecked, the entire vine may die within 10 yr of infection (Pascoe 1999). Foliar symptoms, caused by translocatable toxins produced by *E. lata* in the vascular tissue (Renaud, Tsoupras & Tabacchi 1989, Molyneux *et al.* 2002), include the dwarfing of internodes, leaf tattering and necrosis and the eventual death of infected cordons. These symptoms are clearly visible in spring although they may be confused with other diseases or disorders and quickly become obscured by vigorous growth from neighbouring cordons. Traditionally, diagnosis is dependent upon the identification of the anamorph in culture. However, hyphae of *E. lata* lack unique diagnostic features and production of conidia is erratic (Carter 1991), making it difficult to distinguish *E. lata* from other ascomycetes, in particular other diatrypaceous fungi (Glawe, Skotland & Moller 1982, Rolshausen, Trouillas & Gubler 2004). In addition, other microorganisms present in grapevine wood may grow more rapidly than *E. lata* in culture, resulting in false negative diagnosis.

PCR primers for the detection of *E. lata* in infected wood, based on ITS sequences and sequence characterised amplified regions (SCARs), were reported by Lecomte *et al.* (2000). These primers were used to

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detect DNA of *E. lata* in a supernatant obtained by boiling shavings of diseased grapevine wood in water (Lecomte *et al*. 2000). However, recent research suggests that the ITS-based primers of Lecomte *et al*. (2000) are not, in fact, specific to *E. lata*, but also amplify DNA from other diatrypaceous fungi associated with grapevine (Rolshausen *et al*. 2004). *E. lata*-specific PCR primers have also been developed by Californian researchers (Irelan, Gubler & DeScenzo 1999), however, these primers are the subject of pending patents. Rolshausen *et al*. (2004) developed a PCR-RFLP-based protocol capable of identifying *E. lata* following amplification of DNA using the universal ITS1 and ITS4 PCR primers and digestion of the PCR product with the restriction enzyme *Alu*I. However, this protocol cannot be used to identify *E. lata* directly from DNA isolated from grapevine wood or from mixed fungal cultures, because the universal ITS primers are likely to amplify DNA from other fungi, giving multiple PCR products.

The major objective of this study was to develop specific DNA markers for the rapid, reliable identification of *E. lata* in culture and the detection of the pathogen in infected wood. To achieve this we designed SCAR primers which were derived from sequences of RAPD fragments and constructed a genomic DNA library from which RFLP clones specific to *E. lata* were identified. The SCAR primers of Lecomte *et al*. (2000) were also evaluated.

MATERIALS AND METHODS

Fungal isolates

The isolates of *Eutypa lata* used in this study (Table 1) were obtained from perithecia on mature wood or from hyphae in infected trunks or cordons, as described by Carter (1991), and tentatively identified on the basis of morphology of the anamorph. Cultures were maintained in the dark on potato dextrose agar (PDA, Difco, Sparks) at 25 °C and stored in sterile distilled water at 4 °.

Development of SCAR markers

Extraction of DNA

Isolates were incubated at room temperature for 10–14 d in 250 ml flasks containing 80 ml of liquid medium (20 ml l^{-1} Vogel's medium N (Vogel 1964); 10 g l^{-1} sucrose; 5 g l^{-1} yeast extract). Mycelium was harvested by filtration, rinsed in 300 ml sterile distilled water and stored at −70 °. Total DNA was extracted using methods from Rogers & Bendich (1985) and Péros *et al*. (1996) modified as follows. Ten vol of extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA) were added to 80 mg of finely ground mycelium, mixed by vortexing and incubated at 65 ° for 30 min. An equal vol of phenol: chloroform: *iso*-amyl alcohol (25:24:1) was added, the extract mixed on a rotating disc for 10 min then centrifuged at 12 000 ***g*** for 20 min. The aqueous phase was incubated at 37 ° for 30 min with 10 µl RNase A (10 mg ml^{-1}), then 0.2 vol of 10% CTAB and 0.8 vol of chloroform: *iso*-amyl alcohol (24:1) were added and mixed by inversion. The suspension was centrifuged at 12 000 ***g*** for 15 min, the supernatant transferred to a new microcentrifuge tube then 0.2 vol of 10 M ammonium acetate and 2 vol of ice cold 100% ethanol were added. DNA was precipitated at −20 ° for 2 h then the solution was centrifuged for 10 min at 12 000 ***g***, and the pellet washed in 70% ethanol then resuspended in Tris-EDTA (TE) buffer, pH 8.0. DNA was subjected to electrophoresis in a 0.8% Tris-acetate-EDTA (TAE) gel and quantity was assessed using a λ *Hin*dIII DNA ladder (Roche Diagnostics, Mannheim). DNA concentration was adjusted to approximately 25 ng μl^{-1}.

RAPD-PCR amplification

A total of 86 primers from Operon Technologies (Alameda) RAPD™ 10-mer kits OPA, OPD, OPE, OPJ, OPU and OPAM was initially screened with DNA from *Eutypa lata* isolates M280 and N01. Eighteen primers which resulted in intense bands of the same size from each *E. lata* isolate were screened with DNA from a further 12 *E. lata* isolates and 14 other fungi isolated from grapevine (Table 1). Two DNA fragments, apparently specific to *E. lata*, were selected for cloning and characterisation.

RAPD-PCR amplification reactions were performed in a total volume of 25 µl, using a Corbett Research PC-960 thermocycler. Each reaction contained 0.1 vol of 10 × thermophilic buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% Triton®X-100; Promega Madison, WI), 2 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP (Roche Diagnostics), 30 ng of primer (Operon Technologies), 1 U of *Taq* DNA polymerase (Promega) and approximately 25 ng template DNA. An initial denaturation step of 4 min at 94 ° was followed by 36 cycles of 1 min at 93 °, 1 min at 38 ° and 1 min at 72 °, with a final extension period of 6 min at 72 ° (Péros *et al*. 1996). Amplification products were separated by electrophoresis in 1.2% agarose gels using Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide and visualised under uv illumination. Either a 1 kb ladder (Promega) or a 200 bp DNA ladder (Geneworks, Adelaide) was used as a molecular size marker.

Cloning, characterisation of the RAPD fragments and primer design

Two putative *Eutypa lata*-specific amplicons, one derived from RAPD primer OPAM 02 and the other from primer OPAM 16, were selected from PCR products of each of three isolates (M302, N04 and SS6). Each amplicon was purified from an agarose gel using

Table 1. Origin and source of *Eutypa lata* and other fungal isolates used in this study.

Isolate no.	Species	Host species and origin	Year of isolation	Location
1765[a]	*Botryosphaeria ribis*	*Vitis vinifera*	2000	Victoria
H001[a]	*Cryptovalsa ampelina*	*V. vinifera*, perithecia	2000	Coonawarra, SA
1776[a]	*E. lata*	*V. vinifera*, perithecia	2000	SA
200/1	*E. lata*	*V. vinifera*, mycelium	2002	McLaren Vale, SA
A166[a]	*E. lata*	*V. vinifera*, mycelium	2000	SA
AsW[c]	*E. lata*	*V. vinifera*, perithecia	2000	Barossa Valley, SA
B001[a]	*E. lata*	*V. vinifera*, mycelium	2000	Nuriootpa, SA
B040[a]	*E. lata*	*V. vinifera*, mycelium	2000	SA
Cab2[c]	*E. lata*	*V. vinifera*, perithecia	2000	Barossa Valley, SA
D001[a]	*E. lata*	*Prunus armeniaca*, mycelium	2000	SA
E100[a]	*E. lata*	*V. vinifera*, mycelium	2000	Eden Valley, SA
K001[a]	*E. lata*	*V. vinifera*, mycelium	2000	Knoxfield, Victoria
M266[e]	*E. lata*	*V. vinifera*, perithecia	1999	France
M279[e]	*E. lata*	*Prunus armeniaca*, perithecia	1999	Victoria
M280[e]	*E. lata*	*V. vinifera*, perithecia	1999	Victoria
M295[e]	*E. lata*	*V. vinifera*, perithecia	1999	Victoria
M302[e]	*E. lata*	*V. vinifera*, perithecia	1999	Victoria
M335[e]	*E. lata*	*V. vinifera*, perithecia	1999	Victoria
Ma082	*E. lata*	*V. vinifera*, mycelium	2003	McLaren Vale, SA
Ma114	*E. lata*	*V. vinifera*, mycelium	2003	McLaren Vale, SA
N01	*E. lata*	*V. vinifera*, perithecia	2000	Nuriootpa, SA
N03	*E. lata*	*V. vinifera*, perithecia	2000	Nuriootpa, SA
N04	*E. lata*	*V. vinifera*, perithecia	2000	Nuriootpa, SA
N07	*E. lata*	*V. vinifera*, perithecia	2000	Nuriootpa, SA
N08	*E. lata*	*V. vinifera*, perithecia	2000	Nuriootpa, SA
P #1 SS12[b]	*E. lata*	*Pyrus communis*, perithecia	1981	SA
SS 357-3[b]	*E. lata*	*Genista* sp., perithecia	1984	SA
SS10[b]	*E. lata*	*V. vinifera*, perithecia	1981	SA
SS11[b]	*E. lata*	*V. vinifera*, perithecia	1981	SA
SS2[b]	*E. lata*	*V. vinifera*, perithecia	1981	SA
SS4[b]	*E. lata*	*V. vinifera*, perithecia	1981	SA
SS6[b]	*E. lata*	*Viburnum opulus*, perithecia	1984	SA
E120[d]	*E. lata*	*V. vinifera*, mycelium	Unknown	California
E125[d]	*E. lata*	*V. vinifera, mycelium*	Unknown	Italy
E178[d]	*Diatrype* sp.	*Quercus lobata*, mycelium	Unknown	California
E1[d]	*E. lata*	*V. vinifera*	Unknown	Australia
SS1#1[f]	*Cryptovalsa* sp.	*V. vinifera*, perithecia	1999	New Zealand
1775[a]	*Phaeoacremonium aleophilum*	*V. vinifera*	2000	Victoria
1750[a]	*Phaeomoniella chlamydospora*	*V. vinifera*	2000	Victoria
B041	*Phaeomoniella chlamydospora*	*V. vinifera*	2000	Barossa Valley, SA
1739[a]	*Phellinus punctatus*	*V. vinifera*	2000	Victoria
1740[a]	*Phellinus* sp.	*V. vinifera*	2000	WA
L039	*Phellinus* sp.	*V. vinifera*	2000	Loxton, SA
1785	*Uncinula necator*	*V. vinifera*	1997	SA
C2a	Unidentified ascomycete	*V. vinifera*, perithecia	2000	Eden Valley, SA
C2b	Unidentified ascomycete	*V. vinifera*, perithecia	2000	Eden Valley, SA
EV01	Unidentified ascomycete	*V. vinifera*, perithecia	2000	Eden Valley, SA
EV02	Unidentified ascomycete	*V. vinifera*, perithecia	2000	Eden Valley, SA
EV03	Unidentified ascomycete	*V. vinifera*, perithecia	2000	Eden Valley, SA
W01	Unidentified basidiomycete	*V. vinifera*	2000	Warriparinga, SA
W05	Unidentified basidiomycete	*V. vinifera*	2000	Warriparinga, SA
W07	Unidentified basidiomycete	*V. vinifera*	2000	Warriparinga, SA

SA, South Australia; NSW, New South Wales; and WA, Western Australia.
[a] Isolates provided by Mette Creaser.
[b] Isolates obtained, from the culture collection of Maurice Carter.
[c] Isolates provided by Sharmini John.
[d] Isolates provided by E. & J. Gallo Winery.
[e] Isolates provided by Mary Cole.
[f] Isolate provided by Peter Long.

the Bio-101 Geneclean® II kit (Qbiogene, Carlsbad), then ligated into the pGEM-T® Easy Vector (Promega) and transformed into competent cells of *Escherichia coli* strain JM 109 (Promega) according to the manufacturer's protocol. Plasmid DNA from transformant colonies was isolated using the Wizard® *Plus* SV Minipreps DNA purification system (Promega). The sequence of each amplicon was determined by automated DNA sequencing in forward and reverse directions, using the universal primers M13F and M13R

(Messing 1983). For each amplicon, forward and reverse sequences were compared using the computer package Chromas, version 1.45 (Technelysium, Tawantin) then consensus sequences for the three amplicons derived from each RAPD primer were generated using Clustal W (Thompson, Higgins & Gibson 1994). For each consensus sequence, three SCAR primer pairs were designed using the computer software package OLIGO® Version 6.57 (Molecular Biology Insights, Cascade) and synthesised by Geneworks. Each primer pair was initially validated using *E. lata* isolates M280, N04 and SS6, and then using DNA from nine *E. lata* and two non-*E. lata* isolates previously analysed for secondary metabolite production (Mahoney *et al.* 2003). One primer pair (designated Eut02 F3/Eut02 R2) was then tested with a further 21 *E. lata* isolates and 12 other fungi isolated from grapevine, as well as grapevine DNA. Amplification reactions were conducted in a total volume of 25 µl as outlined above, except that primers were used at a concentration of 0.2 µM each. Amplification of DNA was achieved by an initial denaturation step of 2 min at 94 °, followed by 37 cycles of 30 s at 94 °, 30 s at 65 ° and 1 min at 72 °, with a final extension of 10 min at 72 °.

Evaluation of SCAR markers of Lecomte et al. (*2000*)

The SCAR primer pairs developed by Lecomte *et al.* (2000) were tested on DNA from 24 isolates of *Eutypa lata* and 14 other grapevine inhabiting fungi of Australian origin. Reactions were set up using the conditions described by Lecomte *et al.* (2000), with each reaction performed at least twice.

Cloning and identification of RFLP probes

Approximately 5 µg of DNA from *Eutypa lata* isolate M280 were digested to completion with *Pst*I restriction enzyme and ligated into the plasmid vector pBluescript. Competent cells of *E. coli*, strain JM109, were transformed with 300 ng of ligated DNA and white colonies, presumed to contain recombinant plasmids, were selected and stored in 15% glycerol at −70 ° (Sambrook, Fritsch & Maniatis 1989). Plasmid DNA was isolated from a random selection of 100 clones using the Wizard® *Plus* SV Minipreps DNA purification system. The presence of insert DNA was confirmed by digestion with *Pst*I and the subsequent separation by electrophoresis on 1% agarose gels with TBE buffer. The 100 clones containing inserts were transferred to positively charged nylon membranes (Amersham, Little Chalfont) according to the method of Southern (1975) and cross-linked to membranes using uv light from a GS Gene Linker (Bio-Rad, Hercules). The presence of *E. lata* DNA was confirmed by hybridisation with total radiolabelled DNA from *E. lata* isolate M280. DNA (approx. 50 ng) was labelled with α-^{32}P-dCTP using the random primer method (Feinberg & Vogelstein 1983). Labelled DNA was separated from unincorporated nucleotides in a Sephadex G-100 column, denatured and added to 10 ml hybridisation solution (Sambrook *et al.* 1989). Hybridisation and washing of membranes was accomplished at 65 °, with four 20 min washes in 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS; 0.5 × SSC, 0.1% SDS and 0.2 × SSC, 0.1% SDS, respectively. Membranes were exposed to X-ray film (X-Omat; Kodak, Rochester) at −70 °.

Five clones, estimated to produce a strong or medium-strength hybridisation signal towards DNA from *E. lata* isolate M280, were selected and assessed for their specificity towards the pathogen. These clones were radiolabelled and used as probes towards total DNA (500 ng) from 23 *E. lata* isolates, 14 non-*E. lata* isolates (Table 1) and grapevine. DNA was digested with *Pst*I and transferred to a nylon membrane by Southern transfer.

Identification from mycelium

SCAR primer pair Eut02 F3/Eut02 R2 was used to verify the identity of cultures of *Eutypa lata* from mycelial extracts using the protocol of Hamelin *et al.* (2000). Briefly, a small amount of mycelium was removed from the surface of actively growing cultures on PDA using a 200 µl pipette tip, incubated for 5 min at 100 ° in 100 µl of extraction buffer (0.5 M Tris-HCl, pH 9, 1% Triton® X-100), then cooled on ice for 5 min. A 1 µl aliquot was added to each 25 µl reaction and amplified as described above, with the inclusion of polyvinyl-pyrrolidone (MW 10 000, PVP-10) to a final concentration of 2%. Extracts were initially prepared from mycelium of 26 authenticated *E. lata* isolates and 14 non-*E. lata* isolates (Table 1) and, subsequently, from 80 putative *E. lata* isolates obtained from the cross-sections of trunks of 198 43 yr-old Shiraz grapevines in McLaren Vale, South Australia. These isolates, which were obtained by plating drill shavings taken from the margins of symptomatic tissue onto PDA, were tentatively identified as *E. lata* on the basis of mycelial morphology.

Detection in infected wood

SCAR primer pair Eut02 F3/Eut02 R2 or RFLP probe C68 was used to detect *Eutypa lata* in 1 yr-old cane segments, cuttings or mature grapevine trunks as outlined below. Unless otherwise stated, mycelium of *E. lata* was recovered from each sample as described by Carter (1991).

Detection using SCAR primer pair Eut02 F3/Eut02 R2

For detection using SCAR primers, DNA was isolated from 1 yr-old Cabernet Sauvignon cane segments 12 months after inoculation with suspensions of *Eutypa lata* ascospores (Creaser & Wicks 2002) or with mycelium of *E. lata* isolate M280 (John *et al.* 2004).

Table 2. Sequence data for primers designed for the amplification of DNA from *Eutypa lata*.

Primer pair	Sequence of primer (5′–3′)	Origin of primer pair	Primer length (bp)	Expected product (bp)
Eut02 F1	GCCTTACCGAAATAGACCAA	OPAM 02	21	424
Eut02 R1	ACGAGTGACAAGCGATCAAAT		20	
Eut02 F2	CGGGTAGGGTTAGGTACGGA	OPAM 02	21	636
Eut02 R2	GGCCTTACCGAAATAGACCAA		20	
Eut02 F3	TGGTGGACGGGTAGGGTTAG	OPAM 02	21	643
Eut02 R2	GGCCTTACCGAAATAGACCAA		20	
Eut16 F1	CCGCCAGCTAAGGACATTGAG	OPAM 16	21	198
Eut16 R1	TGATAGCCTGCCGTCATCGTC		21	
Eut16 F2	CGGCAGGCTATCATAGTAGAA	OPAM 16	19	217
Eut16 R2	GGTGAAGTTATGCGCTCAA		21	
Eut16 F3	CGATGACGGCAGGCTATC	OPAM 16	21	212
Eut16 R3	GCGCTCAATGCATGAACTAAC		18	

Various protocols were used to isolate DNA, namely the Bio-101 FastDNA® SPIN kit for soil (Qbiogene), the DNeasy Plant Mini Kit (Qiagen, Hilden), the rapid extraction protocol of Lecomte *et al.* (2000), the CTAB-based protocol of Wolf, Eimert & Ries (1999) and the silica-based protocol of Rott & Jelkmann (2001). Amplification by PCR was carried out in a total volume of 25 μl, containing approximately 30 ng of total DNA, as outlined above. With the exception of the rapid extraction protocol of Lecomte *et al.* (2000), DNA was extracted from 80 mg of grapevine tissue and the quantity of DNA was estimated by comparison with a λ *Hin*dIII DNA ladder as described above.

Detection using RFLP probe C68

Clone C68 was selected to detect *Eutypa lata* in grapevine wood using slot-blot hybridisation. Total DNA was extracted from grapevine tissue (80 mg) using the SEAPS protocol (Melanson, Rawnsley & Scheper 2002). Slot-blots, using DNA extracted from grapevine tissue as well as standard amounts of *E. lata* genomic DNA (1 to 50 ng), were prepared using the Bio-dot SF unit (Bio-Rad) as recommended by the manufacturer. Blots were hybridised and exposed to X-ray film as described above.

The probe was initially tested using DNA isolated from 12 grapevine cuttings (Cabernet Sauvignon), ten of which had been inoculated with mycelium of *E. lata*. DNA was isolated 12 weeks after inoculation of the cuttings. DNA was also isolated from 11 naturally infected trunks (Cabernet Sauvignon) which exhibited wedge-shaped staining characteristic of infection by *E. lata*. Eight DNA samples were isolated from a cross-section of a single diseased trunk (Cabernet Sauvignon), the first sample from the centre of the canker and the remaining seven from asymptomatic wood at locations distributed evenly around the edge of the cross-section. In addition, DNA isolated from discoloured wood in cross-sections of the 198 trunks (Shiraz) obtained from McLaren Vale was analysed.

RESULTS

Development of SCAR markers

The amplicon derived from RAPD primer OPAM 02 was 663 bp long for isolates M302 and N04; however, a 5 bp deletion was identified for isolate SS6. Each amplicon derived from primer OPAM 16 was 505 bp long. Apart from an occasional single base substitution, no differences in sequence were observed among isolates. A search of the GenBank database revealed no significant homology towards any known nucleic acid or protein sequence. Three forward and reverse primer pairs were designed for each consensus sequence; primer sequences and expected fragment sizes are given in Table 2.

Validation of each primer pair using genomic DNA from three isolates of *Eutypa lata* resulted in PCR products of the expected size in each case. However, when tested with the 11 isolates used by Mahoney *et al.* (2003), all primer pairs derived from RAPD primer OPAM 16 detected DNA of either one or both of isolates E178 and SS1#1 (*Diatrype* sp. and *Cryptovalsa* sp., respectively). Hence, these primer pairs were not considered specific to *E. lata*. The primer pairs derived from RAPD primer OPAM 02 did not detect DNA from either of these isolates. Primer pair Eut02 F3/Eut02 R2, derived from RAPD primer OPAM 02, was then tested with genomic DNA from 24 isolates of *E. lata* and 12 other fungi isolated from grapevine. DNA fragments of the expected size (643 bp) were observed for all *E. lata* isolates but not for any other species tested (Fig. 1). This primer pair was, therefore, considered specific to *E. lata* and, consequently, was selected for further PCR-based analyses.

Evaluation of SCAR markers of Lecomte et al. *(2000)*

SCAR primer pair SCB 02A and SCB 02B produced no amplification products when tested with DNA of *Eutypa lata* or any other isolates. Primer pair SCD 18A and SCD 18B generated bands of the expected size (450 bp) in 14 of 24 *E. lata* isolates but no other

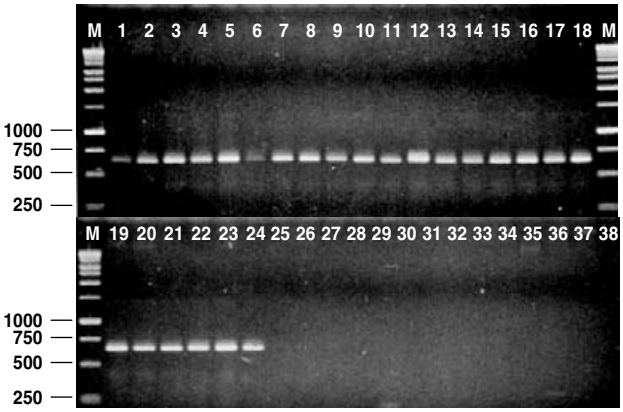


Fig. 1. PCR amplification of DNA from *Eutypa lata* and non-*E. lata* isolates using SCAR primer pair Eut02 F3/Eut02 R2. Lanes 1–24, DNA from *E. lata* isolates M280, M295, M302, N01, N03, N04, N07, N08, AsW, Cab2, SS2, SS4, SS6, SS10, SS11, B001, D001, K001, B040, A166, P#1SS12, E100, 1776, SS357-3; lanes 25–36, DNA from non-*E. lata* isolates C2a, C2b, W01, W05, EV01, 1785, H001, 1739, 1740, 1750, 1765, 1775; lane 37, DNA from grapevine; lane 38, PCR negative control. M, molecular weight marker (1 kb ladder; Promega), size in bp indicated on the left.

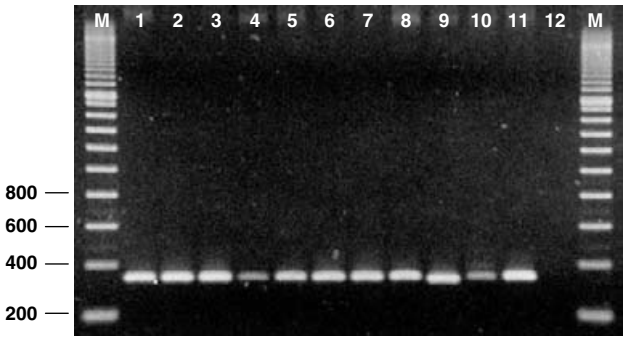


Fig. 2. PCR amplification of DNA from *Eutypa lata* and non-*E. lata* isolates using SCAR primer pair SCA 10A and SCA 10B of Lecomte *et al.* (2000). Lanes 1–11, DNA from isolates E1, E120, E125, E178, M266, M279, M280, M335, N04, SS1#1, SS6; lane 12, PCR negative control. M, molecular weight marker (200 bp ladder; Geneworks), size in bp indicated on the left.

isolates. SCAR primer pair SCA 10A and SCA 10B generated PCR products of the expected size from DNA of all *E. lata* isolates screened, but also for non-*E. lata* isolates E178 and SS1#1 (Fig. 2). Hence, the SCAR primer pairs developed by Lecomte *et al.* (2000) were not considered to be specific to the Australian population of *E. lata*.

Cloning and identification of RFLP probes

We obtained 730 putative recombinant colonies following transformation of *Escherichia coli* with *Eutypa lata* DNA. Of the 100 randomly selected clones, three produced hybridisation signals which were considered strong after X-ray exposure for 5–7 d at −70 °. A further 58 clones displayed a medium-strength signal, 23 a low strength-signal and 16 either a slight or no signal. Of the five clones selected for further analysis, one, designated C68, proved specific to *E. lata*, giving a strong signal when hybridised to DNA from all *E. lata* isolates, but not to any non-*E. lata* isolates or grapevine DNA, after X-ray exposure for 3–10 d at −70 °. A second clone was also specific to *E. lata*, however, signals were faint following X-ray exposure for 10 d at −70 °. The remaining three clones hybridised to DNA from all isolates of *E. lata* as well as to DNA from non-*E. lata* isolates. Consequently, clone C68 was selected for use as an *E. lata*-specific DNA probe. Sequence analysis revealed that clone C68, which was 649 bp long, shared no significant homology with any known nucleotide or protein sequence in the GenBank database.

Identification from mycelium

PCR products of the expected size (643 bp) were present in each *Eutypa lata* isolate but no others when amplifying DNA extracts from mycelium of 26 *E. lata* isolates and 14 non-*E. lata* isolates, using primer pair Eut02 F3/Eut02 R2 (Fig. 3). PCR analysis of mycelium obtained from 198 Shiraz grapevines in McLaren Vale confirmed that 76 of 80 isolates were, in fact, *E. lata*.

Detection in infected wood

Detection using SCAR primer pair Eut02 F3/Eut02 R2

PCR products of the expected size (643 bp) were amplified using DNA isolated from each of 15 inoculated grapevine canes using the Bio-101 FastDNA® SPIN kit for soil. Mycelium of *Eutypa lata* was isolated from each of these samples by culturing on PDA. However, PCR amplification of DNA obtained using the other extraction protocols tested here was inconsistent, often resulting in false negative results even when mycelium of *E. lata* was isolated from the same samples. The failure to obtain PCR-competent DNA using these extraction protocols may be due to inhibition of the reaction by polyphenolic compounds co-purified with DNA. However, whilst the addition of polyvinyl-pyrrolidone (PVP), a compound known to enhance the polymerase chain reaction by binding to polyphenolics (Koonjul *et al.* 1999), to reaction mixtures did enhance the amplification of DNA, false negative results were still common (data not shown).

Detection using RFLP probe C68

Slot-blot assays using DNA isolated from 12 cuttings of cv. 'Cabernet Sauvignon' grown in the glasshouse revealed DNA of *Eutypa lata* in nine of 10 inoculated samples, but not in either of the uninoculated controls, following X-ray exposure for 8 d at −70 ° (Fig. 4). Culturing of wood samples on PDA yielded *E. lata*

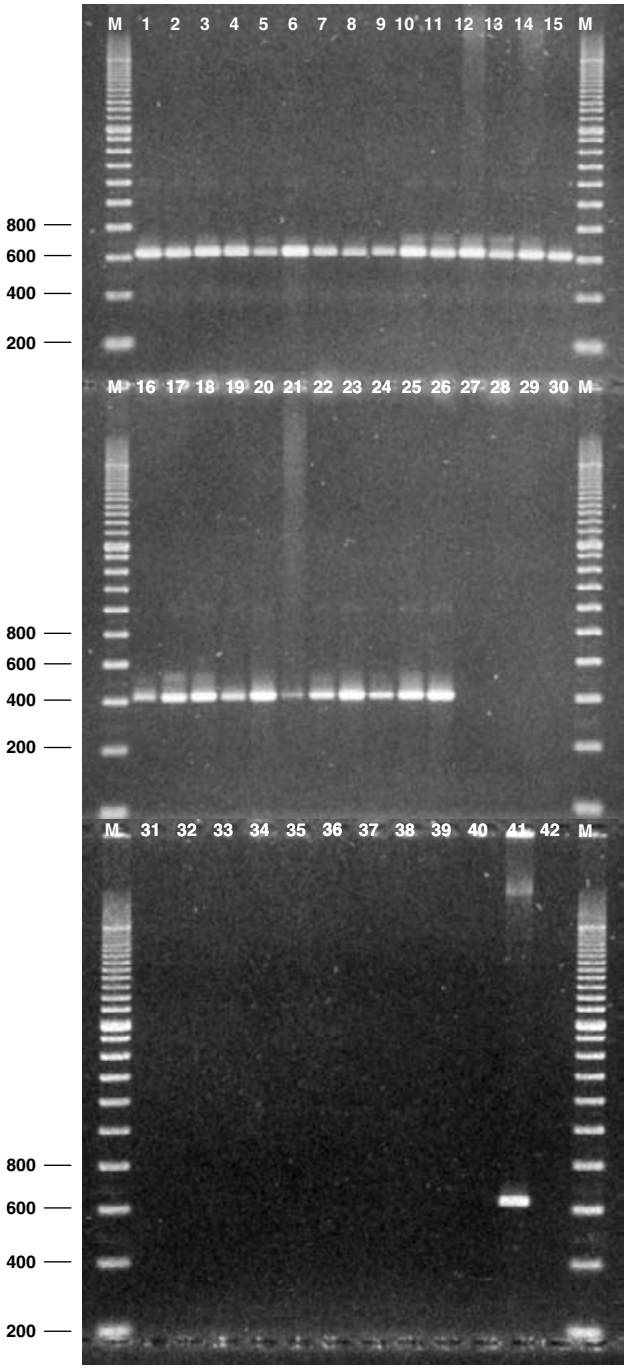


Fig. 3. PCR amplification of *Eutypa lata* DNA isolated from mycelium using the protocol of Hamelin *et al.* (2000), with SCAR primer pair Eut02 F3/Eut02 R2. Lanes 1–26, mycelial extract from *E. lata* isolates M280, M295, M302, N01, N03, N04, N07, N08, AsW, Cab2, SS2, SS4, SS6, SS10, SS11, SS357-3, P#1 SS12, 200/1, E100, D001, B040, K001, B001, 1776, Ma082, Ma114; lanes 27–40, mycelial extracts from non-*E. lata* isolates EV01, EV02, EV03, W01, W05, W07, H001, 1739, 1740, 1750, 1765, 1775, LO39, BO41; lane 41, genomic DNA from *E. lata* isolate M280; lane 42, PCR negative control. M, molecular weight marker (200 bp ladder; Geneworks), size in bp indicated on the left.

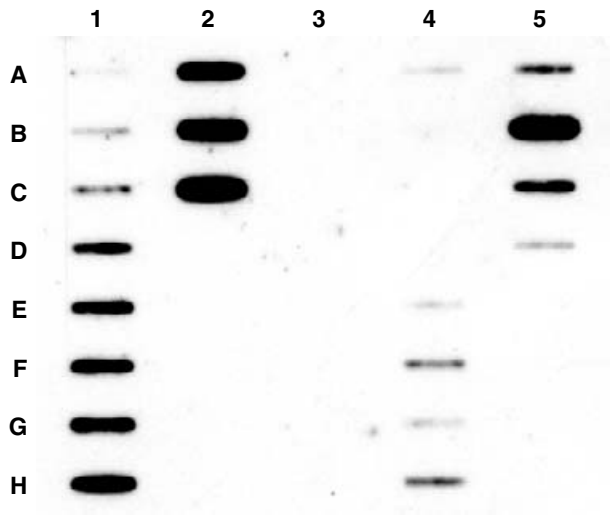


Fig. 4. Detection of *Eutypa lata* in DNA extracted from inoculated canes by slot-blot analysis using the *E. lata*-specific probe C68. Slots 1A–2C contain increasing amounts of genomic DNA from *E. lata* isolate M302 (1, 2.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40 and 50 ng respectively); slots 2D–2H contain dd H_2O; slots 3A–3F contain genomic DNA from grapevine, and from non-*E. lata* isolates 1739, 1740, 1750, 1775, 1785, respectively; slots 3G and 3H contain dd H_2O; slots 4A and 4B, 4E–5D contain 200 ng total DNA extracted from inoculated grapevine canes; slots 4C and 4D contain 200 ng total DNA extracted from uninoculated grapevine canes.

mycelium from only two of the 10 inoculated canes, but not from controls. DNA of *E. lata* was detected in each of the 11 naturally infected trunks (cv. 'Cabernet Sauvignon') which exhibited wedge-shaped staining of the vascular tissue. *E. lata* mycelium was also isolated from each of these trunks. When analysing the cross-section of a single infected trunk, an intense hybridisation signal was apparent for DNA isolated from the centre of the canker and a weaker signal from each of two samples taken from asymptomatic wood at the margin of the canker. No signals were apparent for the remaining five samples which were taken from asymptomatic wood further from the canker. Probe C68 was then used to analyse DNA isolated from each of the 198 trunk cross-sections obtained from cv. Shiraz in McLaren Vale. Slot-blot analysis confirmed the presence of *E. lata* DNA in 107 of the 198 vines (54%, e.g. Fig. 5). Recovery of mycelium from the same samples revealed *E. lata* in 76 of 198 vines (38%), however, various unidentified fungi, which may have obscured mycelium of *E. lata*, were common on these plates.

When using probe C68 to detect DNA of *E. lata* in wood, there was considerable variation in signal strength between extracts, representing different amounts of DNA of *E. lata* present in each total DNA sample extracted from grapevine canes or trunks. For instance, in Fig. 4, slots 4A and 4E, containing DNA extracted from grapevine cane, showed a very weak signal, corresponding to approximately 1 ng of *E. lata* genomic DNA (slot 1A), whereas slot 5B showed an intense signal, corresponding to 40–50 ng of genomic DNA (slots 2B and 2C). Similar variations in signal intensity were observed in samples taken from mature grapevine trunks (e.g. Fig. 5).

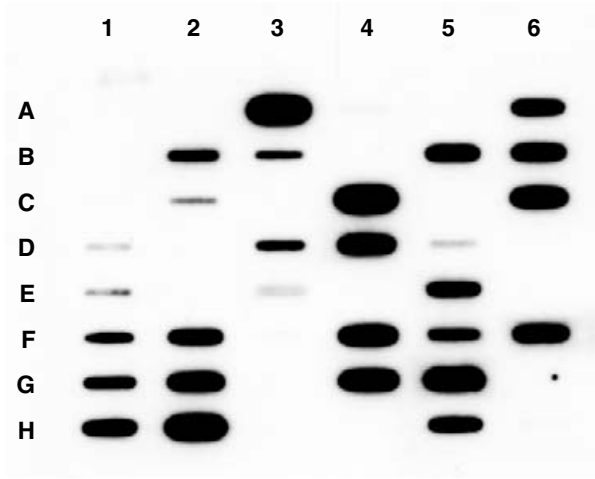


Fig. 5. Example of detection of *Eutypa lata* in DNA extracted from naturally infected grapevine trunks by slot-blot analysis using the *E. lata*-specific probe C68. Slot 1A contains genomic DNA from grapevine; slots 1B–1H contain increasing amounts of genomic DNA from *E. lata* isolate M302 (0, 1, 5, 10, 25, 40 and 50 ng respectively); slots 2A–6H contain total DNA extracted from discoloured wood in grapevine trunk cross-sections.

DISCUSSION

The objective of this study was to develop molecular markers capable of identifying *Eutypa lata* and detecting the pathogen in infected wood. We used two approaches, namely the generation of SCAR primers for use in PCR-based assays and the development of RFLP probes for use in Southern hybridisation-based assays, such as slot-blots. Both types of marker detected DNA from all isolates of *E. lata* tested, but not any other species, and could detect the pathogen in grapevine tissue known to be infected with *E. lata*. DNA markers specific to *E. lata* have been developed previously (Irelan *et al.* 1999, Lecomte *et al.* 2000, Rolshausen *et al.* 2004), however, this is the first report in which Southern hybridisation has been used to detect *E. lata* in infected grapevine wood.

Rolshausen *et al.* (2004) reported that the three ITS-derived primer pairs of Lecomte *et al.* (2000) also detected DNA from related diatrypaceous fungi, a finding supported by analysis of nucleotide sequences of diatrypaceous fungi which have recently been deposited in the GenBank database (Rolshausen *et al.* 2004). Although the markers developed in the current study were not tested against a wide range of diatrypaceous fungi, two isolates, believed to be *Diatrype* sp. (isolate E178, Table 1) and *Cryptovalsa* sp. (isolate SS1#1), were not detected when using SCAR primer pair Eut02 F3/Eut02 R2 or probe C68. Furthermore, a BLAST search of GenBank in August 2004 revealed no homology between DNA of the SCAR fragment and recently deposited sequences of diatrypaceous fungi. Sequence analysis of probe C68 revealed slight homology with one sequence each of *E. maura* and *Eutypella leprosa*, however, in each instance only a single region less than 30 bp long was homologous. Hence, it seems unlikely that this would result in non-specific binding of the probe to DNA of other fungi.

The SCAR markers reported here could detect DNA of *E. lata* in infected wood, although consistent results were achieved only for DNA extracted with the Bio-101 FastDNA® SPIN kit for soil. The failure to amplify DNA extracted using other protocols was considered to be due to inhibition of the PCR by polyphenolic compounds which were co-purified with DNA. Notably, we could not amplify DNA of *E. lata* from infected tissue using the rapid extraction protocol developed by Lecomte *et al.* (2000). Lecomte *et al.* (2000) observed that primer pairs derived from ITS sequences were more efficient in detecting *E. lata* in wood than those derived from RAPD fragments, perhaps because of the high copy number of rDNA targeted by ITS-based primers. RFLP analysis using the SCAR marker derived from RAPD primer OPAM 02 confirmed that this fragment was low copy number (data not shown). However, even when extracts prepared using the protocol of Lecomte *et al.* (2000) were seeded with genomic DNA from *E. lata* isolate M280, amplification by PCR was inconsistent (data not shown). This suggests that the failure to amplify DNA of *E. lata* using SCAR primer pair Eut02 F3/Eut02 R2 was not solely due to the low copy number of the target DNA sequence and was, perhaps, partially due to inhibition of the PCR by compounds present in the grapevine wood. This hypothesis is supported by the observation that the addition of PVP to reaction mixtures did enhance the amplification of DNA, although false negative results were still common. Because the Bio-101 extraction kit was not cost-effective for use in routine identification of *E. lata* in the current study, and because the slot-blot technique allowed reliable detection of DNA of *E. lata* in infected wood, the potential for using other DNA isolation protocols which would permit reliable amplification of DNA by PCR was not further explored here.

DNA of a quality suitable for use in the slot-blot protocol was readily isolated using the SEAPS extraction protocol of Melanson *et al.* (2002). The slot-blot assay was a reliable method for detecting the pathogen in 1 yr old grapevine canes and in mature wood infected with *E. lata*. The variation in signal strength between samples probably reflects the amount of mycelium in the cane or trunk from which the wood was taken. Although when isolating mycelium of *E. lata* from hyphae in dead wood it is preferable to culture material from the margin of healthy and discoloured wood (Carter 1991), results obtained here suggest that when preparing material for slot-blot analysis it is preferable to isolate DNA from dead, discoloured wood. Although total DNA yields were much smaller when isolating from this area, the proportion of DNA from *E. lata* was considerably larger, as shown by the more intense signals from samples obtained from dead wood. Indeed, extraction of DNA from a single infected trunk revealed the strongest signal when samples were taken

from the centre of a canker, with less intense signals from DNA extracted from wood at the margin of the canker. No signals were obtained for DNA extracted from asymptomatic wood more distant from the canker. Given the stronger signals in the samples with least total DNA, this suggests that DNA from the dead grapevine tissue had degraded and little could be isolated, hence, the majority of DNA probably originated from mycelium of *E. lata*.

The analysis of 198 trunks of Shiraz from McLaren Vale, each of which was also assessed for the presence of *E. lata* by plating onto PDA, showed that, in this instance, molecular detection was more sensitive than culturing, illustrating the potential of the *E. lata* specific probe for epidemiological studies. The greater sensitivity of the slot-blot technique may be because *E. lata* mycelium on agar was overgrown by faster growing fungi in the wood, hence its presence could not be detected by visual assessment of colonies on agar, whereas the presence of DNA of other fungi would not influence the outcome of slot-blot assays.

As noted previously, identification of *E. lata* based on cultural characteristics may be ambiguous and incubation for 1–2 months may be required to confirm the identity of the pathogen (Glawe *et al*. 1982, Carter 1991). In the current study, several fungi isolated from perithecia on mature grapevine wood were initially identified as *E. lata* on the basis of morphology of the anamorph and shown to be otherwise following prolonged incubation. The SCAR primers developed here could be used reliably to identify the pathogen from such cultures within 2–3 d when samples were processed according to Hamelin *et al*. (2000), a procedure involving minimal sample preparation. These primers could be used to amplify DNA of mycelial extracts taken from mixed cultures where it is suspected that mycelium of *E. lata* is present but has been obscured by faster growing fungi.

The molecular markers developed in this study will allow the rapid and reliable identification and detection of *E. lata* in grapevine cane or wood. The SCAR markers developed here were more specific than those developed by Lecomte *et al*. (2000), and detected all isolates of *E. lata* used in this study. Furthermore, the SCAR markers developed in this study enable the positive identification of *E. lata* by the amplification of a single DNA fragment, a technique less time-consuming than the PCR-RFLP technique. One drawback of the PCR-RFLP technique is that it requires pure cultures of *E. lata* to enable accurate interpretation of RFLP profiles, whereas the SCAR markers developed in this study could be used to identify the pathogen in mixed cultures, or directly from grapevine wood if appropriate DNA extraction procedures were developed. Whereas the PCR-based protocol requires further development to detect DNA of *E. lata* directly in infected grapevine wood, the DNA probe developed here could reliably detect DNA of *E. lata* in infected wood. Consequently, these markers have potential as research tools to gather information on the epidemiology of the disease and to assess the efficacy of potential control agents. Appropriate disease control measures could then be applied to vines infected with *E. lata*, thereby facilitating improved management of Eutypa dieback.

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Phenolic and heterocyclic metabolite profiles of the grapevine pathogen *Eutypa lata*

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Dedicated to memory of Professor Jeffrey B. Harborne

Abstract

The ascomycete *Eutypa lata* is the causative agent of eutypa dieback in grapevines, a serious economic problem in major wine grape producing areas. In order to develop a predictive, non-destructive assay for early detection of fungal infection, the phenolic metabolite profiles of 11 strains of *E. lata* grown on four different artificial growth media were analyzed by HPLC and their variability compared with growth on Cabernet Sauvignon grapevine wood and wood extracts. Six compounds were generally produced in significant amounts, namely eutypinol, eulatachromene, and eutypine and its benzofuran cyclization product, together with siccayne and eulatinol. The two most widely distributed and abundant metabolites were eutypinol and eulatachromene, which were present in 8 of the strains grown on grapewood aqueous extract fortified with sucrose. Metabolite production on grapevine extract was greatly enhanced relative to the artificial media, indicating that this native substrate provides optimal conditions and a more representative profile of the metabolites produced in the natural disease state. The primary metabolites were tested in a grapeleaf disc bioassay to establish their relative toxicity. Neither eutypinol nor siccayne were phytotoxic; eulatachromene, eulatinol, eutypine, and the benzofuran exhibited necrotic effects in the bioassay. The results indicate that eutypa dieback may be caused by several *E. lata* metabolites rather than a single compound.
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Keywords: *Vitis vinifera*; Vitaceae; Grapes; *Eutypa lata*; Dying-arm disease; Dieback; Eutyposis; Vine decline; Bioassay

1. Introduction

Many major grape-producing areas around the world, including California and Australia, suffer serious economic losses from a fungal infection affecting grapevines (*Vitis vinifera*; Vitaceae) known as eutypa dieback or "dying arm disease. The problem is also known as "eutyposis" after the ascomycete *Eutypa lata* (formerly *E. armeniacae*), which was identified as the causative agent by Moller and Kasimatis (1978). Symptoms of the disease include stunting of new shoots, with small, cupped, chlorotic and tattered leaves, meager development of fruit clusters, and characteristic dark, wedge-shaped necrosis of the trunk and cordons (Fig. 1A and B). Progression of the disease over a period of years results in decreased yields and reduced longevity of the grapevines. Grape cultivars show significant differences in susceptibility to infection, with some of the most valuable, including Cabernet Sauvignon, being particularly sensitive (Munkvold and Marois, 1995). Yield decreases in California for infected Chenin blanc and French Columbard grapes were estimated to range from 30 to 60%, with vineyards over 20 years old having up to 83% reduction, relative to their peak productivity (Munkvold et al., 1994) while in Australia, yield losses

Abbreviations: PCR, polymerase chain reaction; RAPD, Random amplification of polymorphic deoxyribonucleic acid; RFLP, Restriction-fragment length polymorphism.

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for Shiraz and Cabernet Sauvignon vines have been reported as 850 and 740 kg/ha, respectively (Wicks and Davis, 1999). The cost to wine grape production in California has been estimated to be in excess of $260 million per annum, due to decreased yields, increased vineyard management costs and shortened life span of the vines (Siebert, 2000).

E. lata has been isolated from numerous woody species, including grapevines, almond, apricot, cherry, olive, peach and walnut (Carter et al., 1983; Munkvold and Marois, 1994), and from a wide range of non-cultivated hosts (Carter, 1991). The fungus enters the grapevine through pruning wounds, and colonizes the woody tissues. The foliar symptoms may therefore be due to toxins produced by the fungus in the wood and translocated to the shoots. Since foliar symptoms do not become visible until several years after infection, non-destructive procedures for early prediction and removal of infected vines are urgently needed. A PCR-based assay for detecting the fungus in grapevine wood has been developed (Lecomte et al., 2000), but this does not distinguish between pathogenic and non-pathogenic strains, nor provide a measure of the degree of virulence. Methodology based upon the detection in grapevine tissues of the fungal metabolites responsible for phytotoxicity therefore appears to be the best approach for rapid, early diagnosis of the disease.



(a)



(b)

Fig. 1. (A) Symptoms of eutypa dieback in new shoots of grapevine; (B) Necrosis of grapevine trunk wood caused by *Eutypa lata*.

Examination of a laboratory culture of an unspecified strain of the fungus by Renaud et al. (1989a,b) led to the identification of several phenolic compounds bearing a pentenyne side chain *ortho*- to the hydroxyl group, including the benzylic alcohol, eutypinol (**1**), the corresponding aldehyde, eutypine (**3**) and its cyclization product, 2-*iso*propenyl-5-formylbenzofuran (**4**) (Fig. 2), together with highly modified compounds in which the aromatic ring is reduced and the side chain cyclized. Eutypine **3** was postulated as the constituent phytotoxic to grapevines on the basis of in vitro experiments (Tey-Rulh et al., 1991) and was shown to be a protonophoric agent, uncoupling mitochondrial oxidative phosphorylation (Deswarte et al.,1996a,b; Fallot et al., 1997), and acting on chloroplasts (Deswarte et al., 1994) and the plasma membrane (Amborabé et al., 2001). The ability to reduce eutypine **3** to eutypinol **1**, which was not

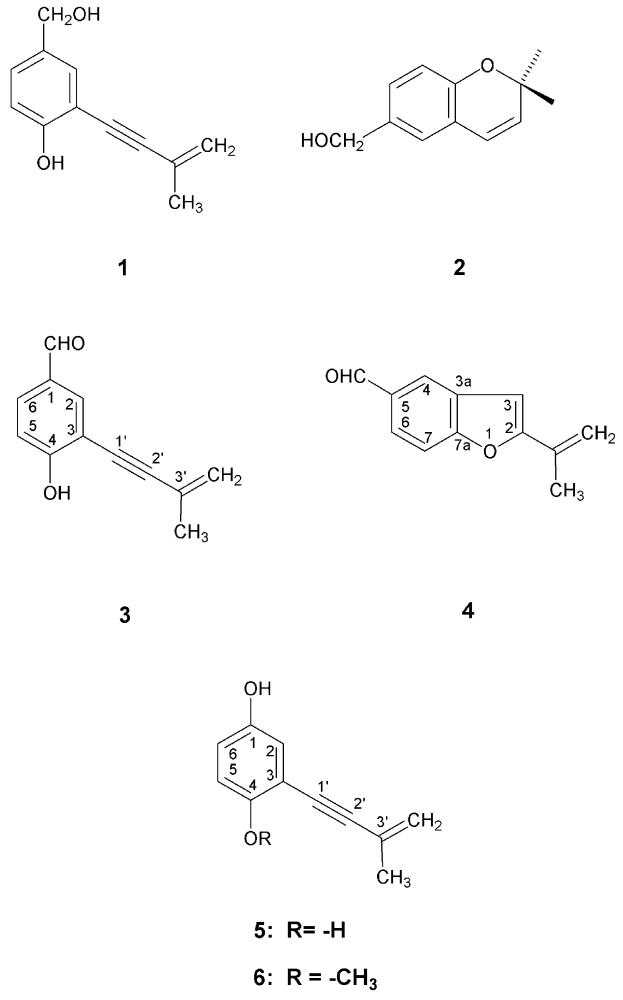


Fig. 2. Chemical structures of *Eutypa lata* metabolites: eutypinol (**1**); eulatachromene (**2**); eutypine (**3**); 2-*iso*propenyl-5-formylbenzofuran (**4**); siccayne (**5**); eulatinol (**6**).

phytotoxic to grape callus tissue in vitro, has been proposed as an explanation for the tolerance of some cultivars to the disease (Guillen et al., 1998). More recently, a comparison of metabolites produced by Californian and Italian strains on malt yeast broth (MYB) and potato dextrose broth (PDB) has identified eulatachromene (**2**), a cyclic analog of eutypinol, and siccayne (**5**) together with its *O*-methyl analog, eulatinol (**6**), as new constituents of *E. lata* (Molyneux et al., 2002); furthermore, eutypine **3** was not produced by the Californian strain even though it has been implicated as being pathogenic to grapevines.

A preliminary structure–activity study (Smith et al., 2003) of synthetic *E. lata* metabolites and analogues, using an in vitro bioassay based on necrosis of grape leaf disks, showed that some phenolic metabolites (**1** and **5**) had no phytotoxic activity, and that eutypine (**3**) had reduced phytotoxic activity compared with the cyclic compounds eulatachromene (**2**) and 2-*iso*propenyl-5-formylbenzofuran (**4**). The variety of metabolites biosynthesized by the fungus, and conflicting information as to those responsible for dieback required that more fundamental information be acquired prior to the use of metabolite detection in grapevines as a diagnostic tool for the disease. This investigation was therefore designed to establish the fungal growth conditions best suited to metabolite production using a number of different strains, especially when grown on grapevine wood and wood extracts, and to identify those metabolites most representative as indicators of fungal infection.

2. Results and discussion

Eleven strains of *E. lata* were chosen for examination, eight of which were originally isolated from grapevines in Australia, California, France, Italy and New Zealand (Table 1). Two strains were from apricot (*Prunus armeniaca*) in Australia and one from Valley oak (*Quercus lobata*) in California. Four of the isolates, E1, E120, E125, and E178, the first from apricot in Australia, the second and third from grape in California and Italy, respectively, and the fourth from oak, have been subjected to genetic analysis using amplified fragment-length polymorphism (AFLP) and the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region (DeScenzo et al., 1999). Although the strains were widely separated geographically, the first three were closely affiliated within a large monophyletic group of 104 strains. In contrast, the oak isolate (E178) was a member of a small, genetically distinct group consisting of four isolates from Valley oak, three isolates from madrone (*Arbutus menziesii*), and only one from grape. This isolate was included as representative of an isolate from a native plant species, with the expectation that its metabolic profile might differ significantly from strains present on the major cultivated plant hosts, grape and apricot, and thus serve as an outlier. The remaining isolates examined in this study had not been genetically screened at the commencement of our experiments on metabolite production.

In an attempt to establish the most productive growth medium for metabolites, all of the *E. lata* strains were initially grown in stationary culture on four artificial liquid media, the defined Vogel's medium (Vogel, 1964; Davis and de Serres, 1970) and that of Pezet (1983), and the complex potato dextrose broth (PDB) and malt/yeast extract broth (MYB). Culture filtrates were extracted after 3–4 week incubation periods, previously shown to be optimal for metabolite production (Molyneux et al., 2002) and the range and amounts of metabolites analyzed by HPLC on a reversed phase column. The standards **1–6** showed baseline resolution with retention times from 16.4–24.3 min (Fig. 3A). Calibration curves were constructed for these major metabolites, either isolated from fungal filtrate (Molyneux et al., 2002) or prepared synthetically (Smith et al., 2003) and characterized by spectroscopic properties and GC–MS, and were linear over a range of 0.2–2.0 μg/20 μl injection. Identity of the peaks was confirmed by retention times and comparison of the UV spectra obtained by photodiode array detection; these spectra provided characteristic distinction between the alcohol, aldehyde, quinol and chromene structural types.

The metabolites identified and their yields are shown in Table 1. It is apparent that the various media supported production of significantly different levels of metabolites and also different profiles, even for the same isolate. In general, the greatest diversity of metabolic products was produced on MYB and Pezet's medium, although the specific compounds varied greatly with fungal strain. In contrast, only three metabolites were produced on PDB (**1**, **5**, and **6**), and Vogel's medium (**1**, **3**, and **6**), respectively, with occasional occurrence of the chromene, **2**. Two of the metabolites from the PDB filtrate, siccayne, **5** and eulatinol, **6**, are obviously structurally related quinols, but this correlation did not extend to the Vogel's medium, which contained no **5**, even though **6** was a common constituent. In fact, siccayne **5** was consistently produced only on PDB, whereas eulatinol **6** was produced by most isolates on all four artificial media. This is somewhat surprising, since **6** would be expected to be produced biosynthetically by methylation of **5**.

The highest amount of metabolites was produced by strain M279, isolated from apricot in Australia, grown on Pezet's medium, which produced 14.7 μg/ml eutypinol (**1**), 1.3 μg/ml eulatachromene (**2**), 0.5 μg/ml eutypine (**3**) and 5.6 μg/ml eulatinol (**6**), together with unidentified peaks that may correspond to *O*-methyl derivatives of eutypinol **1** or eutypine **3**, based on their

Table 1
Metabolite profiles in filtrate of *Eutypa lata* strains grown in four artificial media

Media/metabolite	Metabolite yields (μg/ml) for fungal isolate/source										
	E1 apricot Australia	E120 grape California	E125 grape Italy	E178 oak California	M266 grape France	M279 apricot Australia	M280 grape Australia	M355 grape Australia	NO4 grape Australia	SS1#1 grape New Zealand	SS6 grape Australia
Malt yeast broth (MYB)											
Eutypinol **1**	0.3	0.1	–	–	0.3	0.1	0.1	–	0.1	–	–
Chromene **2**	0.2	–	0.2	0.1	–	–	0.1	–	–	0.1	–
Eutypine **3**	2.5	tr	0.1	tr	3.9	tr	tr	tr	tr	tr	–
Benzofuran **4**	0.1	–	tr	–	0.2	–	–	–	–	–	–
Siccayne **5**	–	0.1	–	–	–	–	0.1	–	–	–	–
Eulatinol **6**	1.1	1.7	2.4	0.2	0.1	–	1.6	0.1	0.1	tr	0.2
Potato dextrose broth (PDB)											
Eutypinol **1**	0.2	–	0.9	–	0.2	0.1	0.1	0.1	0.2	tr	0.1
Chromene **2**	–	–	0.2	–	0.1	–	–	–	–	–	–
Eutypine **3**	–	–	–	–	–	–	–	–	–	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	0.1	0.2	2.0	tr	0.3	tr	0.3	–	0.1	–	tr
Eulatinol **6**	0.1	0.1	0.1	tr	–	0.1	tr	–	0.1	–	tr
Pezet's											
Eutypinol **1**	0.8	0.1	0.2	–	0.7	14.7	0.4	0.2	1.2	0.2	1.3
Chromene **2**	–	–	0.1	–	–	1.3	0.7	–	0.1	0.1	0.4
Eutypine **3**	tr	–	0.1	–	tr	0.5	tr	–	0.1	tr	0.1
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	0.3	–	–	0.1	tr	tr
Eulatinol **6**	–	–	–	–	tr	5.6	0.1	0.1	0.8	0.1	0.1
Vogel's											
Eutypinol **1**	0.8	0.1	0.2	tr	2.2	0.1	0.1	tr	0.1	tr	tr
Chromene **2**	0.1	–	–	–	0.6	0.1	–	–	–	–	–
Eutypine **3**	0.3	tr	0.1	–	0.3	–	tr	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	0.4	1.7	0.2	–	0.1	0.1	2.0	–	tr	–	0.1

– = None detected. tr = < 0.05-detection limit. Detection limits (μg/ml) at 254 nm: **1**, 0.03; **2**, 0.05; **3**, 0.01; **4**, 0.005; **5**, 0.01; **6**, 0.01.

similarities in UV spectra to the latter compounds (Fig. 3B). Surprisingly, all other strains produced less than 0.1 μg/ml of **3** on Pezet's medium, even though this was the medium used for the initial isolation of the compound, although neither its yield or strain of *E. lata* used was specified (Renaud et al., 1989a). Eutypine **3** was produced in significant amounts, 2.5 and 3.9 μg/ml, respectively, only by strains E1 and M266 grown on MYB, accompanied by small amounts of its cyclization product **4**, and was not produced by any of the 11 strains grown on PDB. The benzofuran **4** was not observed for any of the strains grown on PDB, Pezet's or Vogel's media. However, samples that initially contained eutypine **3** showed extensive conversion into the benzofuran **4** on re-analysis, even though the sample had been evaporated to dryness and stored at 0 °C. This confirms our previous observation that eutypine **3** is quite labile and undergoes facile cyclization even when not in solution (Smith et al., 2003). Eutypine **3** was originally proposed as the phytotoxic metabolite of *E. lata* but the low levels produced by most of the strains examined, even on different media, indicate that other metabolites may be involved. Furthermore, we have shown by measurement of chlorophyll reduction in a grapeleaf disk bioassay, that eutypine **3** is approximately 50% less active than the chromene **2** and the benzofuran **4** when tested at a concentration of 50 μg/ml (Smith et al., 2003), suggesting that the latter are more likely to be responsible for the activity.

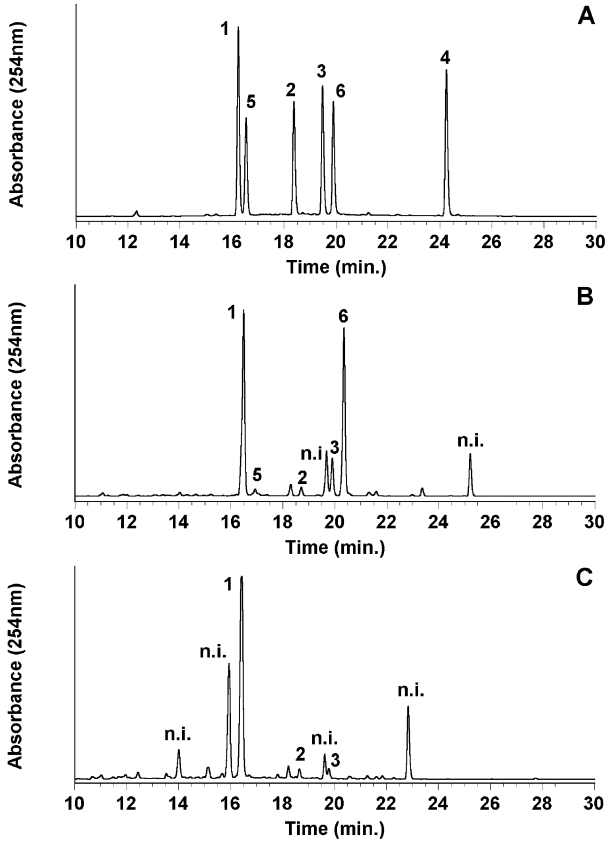


Fig. 3. HPLC analysis of *E. lata* metabolites. (A) Standards **1**–**6**; (B) culture filtrate of isolate M279 grown on Pezet's medium; (C) culture filtrate of isolate E125 grown on grapevine cane aqueous extract + 1% sucrose. n.i. = unidentified metabolites.

The oak isolate, E178, showed little or no metabolite production on any of the four artificial media examined and two grape isolates, M335 from Australia and SS1#1 from New Zealand, showed a similar lack of significant amounts of metabolites. It is therefore probable that not all strains of *E. lata* are capable of inducing foliar symptoms in grapevines and detection of the presence of the fungus is not sufficient evidence to indicate potential for expression of the disease.

In view of the great variability in metabolite production with both media and isolate, it was decided to investigate the effect of growing the fungi on media which more closely approximated the natural situation. The experiments were therefore repeated with inoculation of either finely ground Cabernet Sauvignon cane material or a hot water extract of the cane. Because symptoms of eutypa dieback correlate closely with the Spring flush of growth in the grapevines when sap is freely flowing, the inoculations were also conducted with the same media amended with 1% sucrose. The results of HPLC analysis are shown in Table 2.

The metabolite profiles produced during growth of the fungus on these "natural" media were in marked contrast to those on artificial media, with much more consistent production between strains for each type of medium. The ground cane and cane hot water extracts without added sucrose were relatively non-productive, eutypinol (**1**) being the only metabolite produced at levels above 1 μg/ml, by grape isolates M266 and NO4. Siccayne (**5**) and eulatinol (**6**) were either not produced at all or only at extremely low levels. This was markedly different from the artificial media, on which certain of the strains produced significant amounts, especially of **6**, suggesting that the constituents of grape cane do not favor biosynthesis of the quinol metabolites or else suppress their formation. Previous isolations of siccayne (**5**) from the fungus *Helminthosporum siccans* (Ishibashi et al., 1968) and the marine basidiomycete *Halocyphina villosa* (Kupka et al., 1981) did not report the co-occurrence of acetylenic monophenol metabolites of the eutypinol or eutypine structural type, indicating that the phenol and quinol classes arise from a branching of the biosynthetic pathway.

In contrast, the two sucrose-amended, cane-derived media were especially high in yields of metabolites but these were confined exclusively to eutypinol (**1**), eulatachromene (**2**) and eutypine (**3**); the other three known metabolites were not formed, including the benzofuran (**4**), even when the level of eutypine (**3**) was reasonably high. Unidentified metabolites were also produced, which appeared to be methyl ether derivatives of eutypinol **1** or

Table 2
Metabolite profiles in filtrate of *Eutypa lata* strains grown in "natural" grapevine media with and without sucrose

Media/metabolite	Metabolite yields (μg/ml) for fungal isolate/source										
	E1 apricot Australia	E120 grape California	E125 grape Italy	E178 oak California	M266 grape France	M279 apricot Australia	M280 grape Australia	M355 grape Australia	NO4 grape Australia	SS1#1 grape New Zealand	SS6 grape Australia
Cane hot water extract + 1% sucrose											
Eutypinol **1**	24.7	22.3	29.2	–	30.2	23.1	30.2	0.2	27.5	–	4.7
Chromene **2**	1.5	2.0	2.4	–	0.7	2.8	0.7	–	3.7	–	0.3
Eutypine **3**	0.2	0.1	0.2	–	0.3	5.0	0.8	–	0.1	–	0.2
Benzofuran **4**	–	–	–	–	–	tr	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	–	–	–	–	–	–	–	–	–	–	–
Cane hot water extract/no sucrose											
Eutypinol **1**	0.9	–	0.2	–	0.1	0.2	0.2	–	1.1	–	–
Chromene **2**	–	–	–	–	–	–	–	–	0.5	–	–
Eutypine **3**	tr	–	–	–	–	–	–	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	0.1	0.1	0.1	–	tr	tr	0.1	–	0.1	–	tr
Eulatinol **6**	0.1	–	–	–	–	–	–	–	–	–	–
Ground cane + 1% sucrose											
Eutypinol **1**	1.1	5.1	12.8	–	21.1	0.1	16.3	–	3.0	–	–
Chromene **2**	0.2	0.1	0.5	–	–	–	0.3	–	0.1	–	–
Eutypine **3**	0.1	0.1	0.1	–	0.1	–	0.1	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	–	–	–	–	–	–	–	–	–	–	–
Ground cane/no sucrose											
Eutypinol **1**	0.2	0.1	1.2	–	2.8	–	0.4	–	0.5	–	–
Chromene **2**	–	–	0.1	–	–	–	0.1	–	–	–	–
Eutypine **3**	tr	tr	0.1	–	tr	–	tr	–	tr	–	–
Benzofuran **4**	–	–	–	–	–	–	–	–	–	–	–
Siccayne **5**	–	–	–	–	–	–	–	–	–	–	–
Eulatinol **6**	–	–	–	–	–	–	–	–	–	–	–

– = none detected. tr = < 0.05-detection limit. Detection limits (μg/ml) at 254 nm: **1**, 0.03; **2**, 0.05; **3**, 0.01; **4**, 0.005; **5**, 0.01; **6**, 0.01.

siccayne **5** from the similarities in their UV spectra to the latter compounds; attempts will be made to isolate and elucidate the structures of these constituents from large scale cultures on cane extract medium. The levels of **1** were much higher than those of **2** and **3**, amounting to ca. 30 μg/ml for the grape isolates E125 (Fig. 3C), M266 and M280, from Italy, France and Australia, respectively. As with the artificial media, oak isolate E178 and grape isolates M355 and SS1#1 produced essentially no metabolites. All of the other apricot and grape isolates, except for SS6 from Australia, produced **1** in excess of 20 μg/ml. The second most abundant metabolite was eulatachromene (**2**) with levels generally on the order of 1–4 μg/ml, far in excess of eutypine (**3**) with levels from 0.1 to 0.8 μg/ml. This trend is illustrated in Fig. 4, the only major exception being apricot isolate M279 with 5.0 μg/ml of **3** compared to 2.8 μg/ml of **2**. Previous results have shown that **2** is more phytotoxic than **3**, based on measurement of chlorophyll loss in a grape leaf disk bioassay (Smith et al., 2003). The relative activity at 50 μg/ml for the primary metabolites **1**–**6** in the current study are illustrated in Fig. 5. It is noteworthy that the most active metabolites are eulatachromene (**2**) and the benzofuran (**4**), rather than the acetylenic phenols. Castañeda et al. (1996, 1998) have shown that the acetylchromenes, encecalin and demethylencecalin, and the benzofuran, euparin, isolated from the roots of *Helianthella quinquenervis,* suppress radicle growth of Prince's feather (*Amaranthus hypochondriacus*) and barnyard grass (*Echinochloa crusgalli*) and inhibit photophosphorylation and electron transport in isolated spinach chloroplasts. In addition, the benzofuran (**4**) is the dehydroderivative of fommanoxin [(±)-5-formyl-2-isopropenyl-2,3-dihydrobenzofuran], a metabolite of the basidiomycete *Heterobasidion annosum*, a common wood rot fungus affecting conifers. Fommanoxin has been shown to inhibit protein biosynthesis in *Picea abies* and *Nicotiana tabacum* protoplasts (Sonnenbichler et al., 1989) and cause growth retardation in *Pinus sylvestris* (Hirotani et al., 1977). The compound has been isolated from the stained stem heartwood zone of affected Sitka spruce trees and is toxic to germinating seeds and young seedlings (Heslin et al., 1983). The structural analogies of these compounds with the cyclic *E. lata* metabolites **2** and **4**, suggests that the latter may have a similar mode of action.

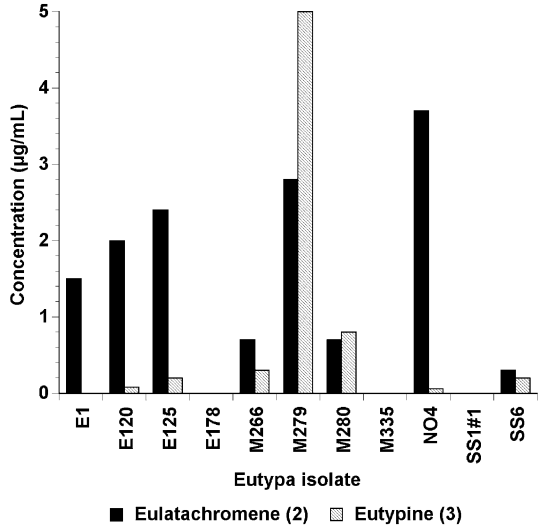


Fig. 4. Comparison of eulatachromene (**2**) and eutypine (**3**) yields for 11 *Eutypa lata* isolates grown on grapevine cane aqueous extract + 1% sucrose.

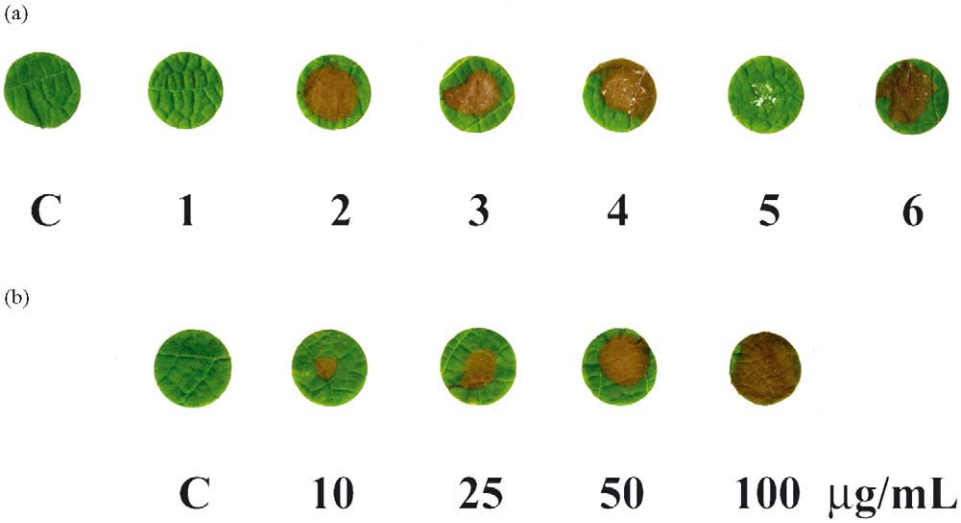


Fig. 5. (A) Comparison of phytotoxicity at 24 h at 50 μg/ml for methanol control (**C**) and the primary metabolites **1–6** in grapeleaf bioassay; (B) comparison of phytotoxicity at 24 h for methanol control (**C**) and eulatachromene (**2**) at 10, 25, 50 and 100 μg/ml.

3. Conclusions

The great variability in metabolite profiles and yields indicates that isolates cultured on artificial media are not useful for establishing the likelihood of any specific *E. lata* isolate producing phytotoxic metabolites in a field situation. However, grape cane hot water extract produced consistent profiles and high levels of metabolites in a medium that best approximates under laboratory conditions the environment available to the fungus during expression of eutypa dieback in planta. We therefore intend to concentrate on use of this medium in the future for examination of additional isolates and to evaluate comparative resistance of different grape cultivars to the disease.

The consistent pattern of metabolites observed for *E. lata* isolates from different plant hosts and locations indicates that these isolates are substantially the same and confirms that eutyposis, as defined on the basis of symptoms, is not a different disease in different geographical areas of the world. Nevertheless, the essential absence of metabolites from isolates E178, M355 and SS1#1 demonstrates that not all *E. lata* strains are likely to be capable of producing characteristic foliar symptoms. This is supported by preliminary results from genetic analysis of the 11 isolates, showing that 9 of them had similar profiles when screened with 10 RAPD primers but that E178 and SS1#1 were consistently different from the rest and from each other; similar results were obtained with RFLP analysis (R. Lardner, unpublished results). Examination of asci produced by SS1#1 has confirmed that it is not *E. lata* (P. Long, personal communication), whereas E178 was previously shown to differ from the majority of *E. lata* strains examined, using AFLP and ITS sequence analysis (DeScenzo et al., 1999). As a consequence, mere detection of the fungus in a grapevine is not sufficient diagnostic evidence for the disease and chemical methods for detection of metabolites are likely to be more suitable. The higher levels produced in grapevine medium, and greater activity of eulatachromene (**2**) than eutypine (**3**) in our bioassay, suggests that the former should be the better diagnostic constituent. However, the much higher levels of eutypinol (**1**) than any other metabolites for all of the producing isolates examined in this study indicates that this compound would be most readily detected in infected tissue, even though bioassay results show that it lacks phytotoxicity. It must be recognized that inactive **1** could serve as a precursor for transformation into active **2** in plant tissue through a simple reduction of the acetylenic bond of the side chain and subsequent cyclization. Future experiments will focus on detection of specific metabolites present in emerging shoots at a time when signs of infection and disease expression are noticeable, and pathogenicity testing of isolates of *E. lata*.

4. Experimental

4.1. Reagents

Pyridine (Fisher Scientific) was dried by storage over molecular sieves, Type 4A, 8-12 mesh (Aldrich Chemical Co.). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) in 1 ml ampules was obtained from Pierce (Rockford, IL).

4.2. General

UV spectra were measured at 190–500 nm in MeOH solution, using a Hewlett-Packard 8452A diode array spectrophotometer. GC–MS analyses were performed on a Hewlett-Packard 5890 Series II instrument equipped with a 5971 mass-selective detector (MSD). HPLC analyses were run on an Agilent 1100 system with detection either by UV at 254 nm (Agilent 1100 variable wavelength detector) or photodioarray at 190–400 nm (Agilent 1100 diode array detector).

4.3. Microorganisms and culture conditions

The fungal isolates were obtained from the E. & J. Gallo Winery Culture Collection (Modesto, CA) (E1, E120, E125, E178); from Drs. Maurice Carter (SS6), Mary Cole (M280, M335, M266, M279) and Peter Long (SS1#1) (Australia); and a collection by R. Lardner (N04). *E. lata* isolates were grown on potato dextrose agar (Difco) and cut into plugs for use as inoculum in all tested media.

4.3.1. Preparation of artificial media

Tested media comprised 2 complex and 2 defined media. Complex media were potato dextrose broth (PDB, Difco) and malt extract/yeast extract (MYB;10 and 20 g/l, respectively; Difco). Defined media were Vogel's Media N (VMN) and Pezet's. VMN consisted of the following nutrients per liter: glucose, 10 g; sodium citrate.$5H_2O$, 3 g; KH_2PO_4, 5 g; NH_4NO_3, 2 g; $MgSO_4.7H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.1 g; biotin, 5 ml of a 5% solution in 50% ethanol; and 5 ml of a trace element solution containing (per 100 ml) citric acid.$1H_2O$, 5 g; $ZnSO_4.7H_2O$, 5 g; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 1 g, $CuSO_4.5H_2O$, 250 mg; $MnSO_4.1H_2O$, 50 mg; H_3BO_3, 50 mg; and $Na_2MoO_4.2H_2O$, 50 mg. Pezet's medium consisted of the following nutrients per liter: glucose, 10 g; sucrose, 5 g; KNO_3, 5 g; KH_2PO_4, 1 g; NaCl, 0.5 g; $CaCl_2.2H_2O$, 0.1 g; $FeCl_3$, 0.05 g; and 1 ml of a trace element solution containing (per 100 ml) $CuSO_4$, 10 mg; $MnSO_4$, 2 mg; H_3BO_3, 1 mg; $ZnSO_4$, 2 mg; $(NH_4)_2MoO_4$, 2 mg; and pH adjusted between 5.5 and 6.0 with 1 N NaOH or 1 N HCl before sterilization.

4.3.2. Preparation of grape-based media

Tested media comprised grape cane and the hot water extract of grape cane. Dormant Cabernet Sauvignon canes (Napa Valley, CA; 2000) were ground in a Wiley mill with a 1 mm screen. The Cabernet cane based medium consisted of 10 g ground cane with 50 ml H_2O; cane medium supplemented with sugar also contained 1 g sucrose per 10 g cane. Liquid cane-based media was prepared by sonifying (Branson) 100 g ground Cabernet cane with 500 ml hot water (100 °C). This extract was clarified by filtration though the following series of filters: Miracloth (CalBiochem), multigrade GMF 150 (Whatman), GF/F (Whatman), and Supor-200 0.2 μm (Gelman). Cane extract supplemented with sugar also contained 1 g sucrose per 50 ml extract.

4.3.3. Inoculation and incubation of cultures

Liquid media (50 ml per 250 ml flask) and cane media (10 g cane per 250 ml flask) were autoclaved followed by the addition of several plugs (6.5 mm diameter) of *E. lata* from margins of the inoculum. All cultures were incubated at 25 °C in the dark for 30 days except for those isolates grown in Vogel's media, which were incubated for 24 days.

4.4. Quantitative analysis of metabolites in Eutypa growth media

Liquid cultures (50 ml) of *E. lata* were filtered through Whatman #4 paper and the filtrate extracted with diethyl ether (2×50 ml). The ether extracts were combined and partitioned with H_2O (50 ml). The organic phase was collected and the ether removed under reduced pressure. The residue was dissolved in methanol (1 ml) and filtered through a 0.45 μm 13 mm syringe filter (Gelman). Cane-based media was extracted by sonification with $CHCl_3$ (50 ml) and filtered though Whatman No. 4 paper. The extract was partitioned with H_2O (50 ml) and the organic phase collected with the $CHCl_3$ removed under reduced pressure. The residue was diluted and filtered as above. Samples (20 μl) were analyzed by HPLC using a Microsorb C_{18} 5 μm column, 250×4.6 mm i.d. (Varian) with a gradient elution at 1.0 ml/min of 100% H_2O with 0.5% HOAc to 100% CH_3CN over 30 min and held at 100% CH_3CN for 5 min. Detection was either by UV at 254 nm or photodioarray. Metabolite concentrations were determined by reference to the standard curves prepared for each compound, which were linear over the range tested, 0.2–20 μg/20 μl injection.

4.5. GC/MS analysis of metabolites

The GC/MS method for analysis of fungal extracts and metabolites as their trimethylsilyl ether (TMS) derivatives has been reported previously (Molyneux et al., 2002). In brief, the sample in dry pyridine was treated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and the resulting TMS derivatives separated on a temperature programmed, 60 m×0.32 mm i.d. SE-30 fused Si capillary column, with on-column injector. The MS was operated at 70 eV in the EI mode with scanning from 75 to 600 a.m.u at a sampling rate of 1.5 scans/s.

4.6. Phytotoxicity to grape leaf disks

Leaf disks (1 cm diameter) were cut from greenhouse grown Cabernet Sauvignon grape leaves (4–5 cm at the widest point) with a cork borer with the major veins being avoided. Ten leaves were used for each concentration such that each of the tested compounds and its associated control had one leaf disk from each of the same ten leaves. The ten leaf disks for each sample were arranged around the perimeter of filter paper (7.0 cm Whatman No. 3) saturated with H_2O (2 ml) and placed in a Petri dish (100×15 mm). Compounds to be tested were dissolved in MeOH and a volume of 5 μl applied as a single spot at the center of the surface of each leaf disk; the control disks were similarly treated with 5 μl of MeOH. The Petri dishes were covered with lids and the test samples exposed at room temperature to ambient lighting on a laboratory bench; this consisted of approximately 12 h of mixed natural and artificial light and 12 h of darkness. An additional volume of H_2O (1.5 ml) was added to each Petri dish after 24 h.

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APPENDIX 2: INTELLECTUAL PROPERTY

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APPENDIX 4: STAFF

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APPENDIX 5: ACKNOWLEDGEMENTS

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APPENDIX 6: OTHER RELEVANT MATERIAL

Table A6.1. Origin and source of *E. lata* and other fungal species used develop DNA markers, for analysis of secondary metabolite production by *E. lata* and for assessing pathogenicity in the shadehouse.

Isolate No.	Species	Source	Year of isolation	Origin
1765	*Botryosphaeria ribis*	*Vitis vinifera*	2000	Victoria
1735	*Cryptovalsa ampelina*	Perithecia, *V. vinifera*	2000	Eden Valley, SA
01017B	*E. lata*	*V. vinifera*	2001	NSW
1776	*E. lata*	Perithecia, *V. vinifera*	2000	SA
200/2	*E. lata*	Mycelium, *V. vinifera*	2002	McLaren Vale, SA
200/3	*E. lata*	Mycelium, *V. vinifera*	2002	McLaren Vale, SA
83330[b]	*E. lata*	*Prunus persica*	1983	SA
83339[b]	*E. lata*	Mycelium, *Quercus* sp.	1983	SA
A166	*E. lata*	Mycelium, *V. vinifera*	2000	SA
AsW[c]	*E. lata*	Perithecia, *V. vinifera*	2000	Barossa Valley, SA
Bx 1–10[d]	*E. lata*	*V. vinifera*	1990	France
CAB2[c]	*E. lata*	Perithecia, *V. vinifera*	2000	Barossa Valley, SA
E 120[e]	*E. lata*	Mycelium, *V. vinifera*	Unknown	California
E 125[e]	*E. lata*	Mycelium, *V. vinifera*	Unknown	Italy
E 178[e]	*E. lata**	Mycelium, *Quercus lobata*	Unknown	California
E 1[e]	*E. lata*	*V. vinifera*	Unknown	Australia
EL3	*E. lata*	*V. vinifera*	Unknown	South Africa
EL4	*E. lata*	*V. vinifera*	Unknown	South Africa
EVS	*E. lata*	Mycelium, *V. vinifera*	2000	Eden Valley, SA
HT01	*E. lata*	Mycelium, *Prunus armeniaca*	2000	SA
IM 334[d]	*E. lata*	*V. vinifera*	1996	Italy
JB16	*E. lata*	Mycelium, *V. vinifera*	2000	SA
Knox	*E. lata*	Mycelium, *V. vinifera*	2000	Knoxfield, Vic
M266[f]	*E. lata*	Perithecia, *V. vinifera*	1999	France
M279[f]	*E. lata*	Perithecia, *P. armeniaca*	1999	Victoria
M280[f]	*E. lata*	Perithecia, *V. vinifera*	1999	Victoria
M295[f]	*E. lata*	Perithecia, *V. vinifera*	1999	Victoria
M302[f]	*E. lata*	Perithecia, *V. vinifera*	1999	Victoria
M335[f]	*E. lata*	Perithecia, *V. vinifera*	1999	Victoria
N01	*E. lata*	Perithecia, *V. vinifera*	2000	Nuriootpa, SA
N03	*E. lata*	Perithecia, *V. vinifera*	2000	Nuriootpa, SA
N04	*E. lata*	Perithecia, *V. vinifera*	2000	Nuriootpa, SA
N07	*E. lata*	Perithecia, *V. vinifera*	2000	Nuriootpa, SA
N08	*E. lata*	Perithecia, *V. vinifera*	2000	Nuriootpa, SA
Nuri	*E. lata*	Mycelium, *V. vinifera*	2000	Nuriootpa, SA
Pear #1 SS12[b]	*E. lata*	Perithecia, pear	1981	SA
PP 496[d]	*E. lata*	*V. vinifera*	1999	Spain
RB 440[d]	*E. lata*	*V. vinifera*	1997	France
SS 357–2[b]	*E. lata*	Perithecia, *Genista* sp.	1984	SA
SS 357–3[b]	*E. lata*	Perithecia, *Genista* sp.	1984	SA
SS1#1[g]	*E. lata**	Perithecia, *V. vinifera*	1999	New Zealand
SS1#3[g]	*E. lata**	Perithecia, *V. vinifera*	1999	New Zealand
SS1#9[g]	*E. lata**	Perithecia, *V. vinifera*	1999	New Zealand
SS10[b]	*E. lata*	Perithecia, *V. vinifera*	1981	SA
SS11[b]	*E. lata*	Perithecia, *V. vinifera*	1981	SA
SS2[b]	*E. lata*	Perithecia, *V. vinifera*	1981	SA
SS4[b]	*E. lata*	Perithecia, *V. vinifera*	1981	SA
SS6[b]	*E. lata*	Perithecia, *Viburnum opulus*	1984	SA
1739	*Phellinus punctatus*	*V. vinifera*	2000	Victoria
1740	*Phellinus sp.*	*V. vinifera*	2000	WA
1750	*Phaeomoniella chlamydosporum*	*V. vinifera*	2000	Victoria
1775	*Phaeoacremonium aleophilum*	*V. vinifera*	2000	Victoria
1780[h]	*Phomopsis viticola*	*V. vinifera*	2001	Adelaide Hills, SA
1785[a]	*Erisyphe necator*	*V. vinifera*	1997	SA
C2	unidentified	Perithecia, *V. vinifera*	2000	Eden Valley, SA

Isolate No.	Species	Source	Year of isolation	Origin
	ascomycete			
C2a	unidentified ascomycete	Perithecia, *V. vinifera*	2000	Eden Valley, SA
EV01	unidentified ascomycete	Perithecia, *V. vinifera*	2000	Eden Valley, SA
EV02	unidentified ascomycete	Perithecia, *V. vinifera*	2000	Eden Valley, SA
EV03	unidentified ascomycete	Perithecia, *V. vinifera*	2000	Eden Valley, SA
W01	unidentified basidiomycete	Perithecia, *V. vinifera*	2000	Warriparinga, SA
W05	unidentified basidiomycete	Perithecia, *V. vinifera*	2000	Warriparinga, SA
W07	unidentified basidiomycete	Perithecia, *V. vinifera*	2000	Warriparinga, SA
B001	*E. lata*	*V. vinifera* (Shiraz)	2000	Barossa Valley, SA
B002	*E. lata*	*V. vinifera* (Shiraz)	2000	Barossa Valley, SA
B003	*E. lata*	*V. vinifera* (Shiraz)	2000	Barossa Valley, SA
B005	*E. lata*	*V. vinifera* (Shiraz)	2000	Barossa Valley, SA
C001	*E. lata*	*V. vinifera* (Cab Sauv)	2000	Clare, SA
C002	*E. lata*	*V. vinifera* (Chardonnay)	2000	Clare, SA
C003	*E. lata*	*V. vinifera* (Riesling)	2000	Clare, SA
L001	*E. lata*	*V. vinifera* (Cab Sauv)	2000	Loxton, SA
L002	*E. lata*	*V. vinifera* (Chenin Blanc)	2000	Loxton, SA
L003	*E. lata*	*V. vinifera* (Chenin Blanc)	2000	Loxton, SA
MA002	*E. lata*	*V. vinifera* (Shiraz)	2003	McLaren Vale, SA
MA019	*E. lata*	*V. vinifera* (Shiraz)	2003	McLaren Vale, SA
MA076	*E. lata*	*V. vinifera* (Shiraz)	2003	McLaren Vale, SA
MA089	*E. lata*	*V. vinifera* (Shiraz)	2003	McLaren Vale, SA
MA101	*E. lata*	*V. vinifera* (Shiraz)	2003	McLaren Vale, SA
MA176	*E. lata*	*V. vinifera* (Shiraz)	2003	McLaren Vale, SA
OR008	*E. lata*	*V. vinifera* (Shiraz)	2003	Coonawarra, SA
OR019	*E. lata*	*V. vinifera* (Shiraz)	2003	Coonawarra, SA
OR022	*E. lata*	*V. vinifera* (Shiraz)	2003	Coonawarra, SA
OR024	*E. lata*	*V. vinifera* (Shiraz)	2003	Coonawarra, SA
OR039	*E. lata*	*V. vinifera* (Shiraz)	2003	Coonawarra, SA
WB031	*E. lata*	*V. vinifera* (Shiraz)	2003	Eden Valley, SA
WB052	*E. lata*	*V. vinifera* (Shiraz)	2003	Eden Valley, SA
WB066	*E. lata*	*V. vinifera* (Shiraz)	2003	Eden Valley, SA
WB115	*E. lata*	*V. vinifera* (Shiraz)	2003	Eden Valley, SA
WB182	*E. lata*	*V. vinifera* (Shiraz)	2003	Eden Valley, SA

South Australia (SA), New South Wales (NSW), Victoria (Vic), Western Australia (WA)

a Isolate provided by Dr Belinda Stummer
b Isolates obtained from the culture collection of Dr Maurice Carter
c Isolates provided by Dr Sharmini John
d Isolates provided by Dr Jean–Pierre Péros
e Isolates provided by E. & J. Gallo Winery
f Isolates provided by Dr Mary Cole
g Isolates provided by Dr Peter Long
h Isolate provided by Dr Belinda Rawnsley
* Indicates isolate was received as *E. lata*, but subsequently shown to be a species other than *E. lata*

APPENDIX 7: BUDGET RECONCILIATION