UNIVERSITY
OF TASMANIA

Novel application of methods to investigate epidemiology and management of botrytis bunch rot in wine grapes

by
Katie Jane Dunne

(Ba Applied Science (Viticulture) (Honours) (Ba Applied Science (Viticulture))
Charles Sturt University

School of Land and Food

Submitted in fulfilment of the requirements for the Doctorate of Philosophy
University of Tasmania, May 2014

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Katie J Dunne
University of Tasmania
May 2014

Authority of Access

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Katie J Dunne
University of Tasmania
May 2014

Acknowledgement

I would not have succeeded in the completion of this PhD if it was not for the help and support from the many people that been part of this journey with me. A rather long journey was like a roller coaster with a few roadblocks thrown in.

Firstly, I would like to thank the wine industry that via their levies enabled the Grape and Wine Research and Development Corporation to fund my research project. I would also like to thank the Royal Hobart Wine show committee for the wine scholarship in which I received in 2010, enabling the purchase of a much-needed computer that would handle all the GIS maps and large files that were created along the way. It was instrumental in putting the final touches together for the award-winning poster at the 14th AWITC.

My PhD supervisors, who are spread across two states and continents for their perseverance and guidance. Dr Kathy Evans for her ideas, support during the final thesis preparation and leading the whole Botrytis Project. Dr Karen Barry for providing a plant pathologist view from outside of the wine industry, data analysis ideas, and early stages of editing. Dr Jacky Edwards for her support, expertise and editing, and for enabling me to have the belief that I could complete the project. Finally Dr Lance Cadle-Davidson for the initial invitation to the USDA to learn all things qPCR, letting me take it to the next level, and editing the molecular component of this project.

At the University of Tasmania, I would like to thank the following people:-
All who used the Food Microbiology Laboratory for putting up with my messy and loud grape grinding method. Sorry: - we have since found a better alternative.
Adam Smolenski for enabling the use of bench space, and the equipment in the Central Sciences Molecular Laboratory.
Dr Alison Dann for her help in solving the PCR hurdles and support when the times were rather difficult.

Dr Shane Powell for her help with trouble shooting the initial qPCR issues that arose rather unexpectedly.

Dr Bob Dambergs (UTAS/AWRI) for his expertise in NIR/MIR and PCA analysis. Without the help the help of Justin Direen, the field trials would not have run as smoothly for the two seasons I was in Tasmania. All admin staff (Moya Sue and company) at TIA/ School of Agriculture for processing purchase orders and all other bits and pieces that required forms.

Fellow PhD student Hannah Thompson who I shared an office with and helped with escaping from time to time- our coffee trips, lunch escapades, introducing her to the delights of red wine, and her help during the whole of block experiment.

The following outside of UTAS:-

Dr Rob Bramley (CSIRO) for his expertise, collaboration, and time on the use of Precision Viticulture as a tool in understanding disease epidemics in vineyards. Without access to vineyards, this project would have been possible, to all the growers' thank you.

A special mention goes to Matt Barwick and company at Clarence House for letting me take over the Chardonnay block, especially for the whole of block experiment, which had a few winemakers worried. Alix Bramaude Du Boucheron, Val and various people who have helped me complete my work along the way.

I would also like to thank Dr Fabrice Magnino from Integrated Sciences for his time and help in the design and optimisation of the new qPCR assay.

Mark McClean & Fiona Thomson (DPI Victoria- via Jacky) for providing information on AUDPC.

I would also like to thank my colleagues at Treasury Wine Estates for their support and allowing time for me to complete the final stages of editing of my thesis.

Finally I would like to say a big thank you to all the people I have met who have supported me and kept me going, to my Tassie adopted family David and the late Denise Featherstone.

To my family, especially my nephews and nieces that provided the must needed but exhausting distractions.

To my parents, whom of which have supported me along this journey through the difficulties, that have occurred, my random sleeping hours and moods. A big thank you, I would not have succeeded if it were not your support and patience.

THANK YOU- We all can finally open up that bottle of wine !!!

Abstract

Botrytis bunch rot (BBR) of grapes caused by the fungus *Botrytis cinerea*, can cause yield and quality implications. This study investigated the epidemiology and management of BBR in a cool climate, specifically in the Coal River Valley and Rokeby regions of Tasmania. Field trials were part of a larger project investigating spray timing and risk factors associated with BBR.

Currently there is a shift to develop and use novel methods in the study of BBR epidemiology because they have the potential to provide assessment of total infection of the disease and not just visible disease. Symptoms may not be evident until fruit ripening, even though infection may have occurred weeks or months earlier. As part of this project, a duplex qPCR technique was developed based on a previously published qPCR technique targeting the intergenic region of the *B. cinerea* sequence. The assay was developed specifically to use on wine grapes with the internal control targeting *Vitis vinifera* DNA. The assay was modified and adapted to suit laboratory equipment available and then used to detect and quantify *B. cinerea* DNA from total nucleic acids extracted from grape berry samples.

A study, using qPCR and visual assessment, was conducted during the 2008-09 season to track natural infections of *B. cinerea* in grape berries sampled from commercial vineyards in the period pre-bunch closure until harvest. Temporal progress curves of disease severity were generated using data from two *V. vinifera* cultivars: Riesling and Sauvignon Blanc from different vineyards. The qPCR results confirmed that infection and colonization of the fruit occurs during the early stages of berry development, followed by a latent period. Disease expression during fruit ripening increased with time.

The latent infection pathway was determined to be more important than the necrotic tissue pathway, in a small plot trial conducted in 2007-08. Treatments included four different spray programs with and without removal of bunch trash (decaying floral parts which include calyptras and aborted berries). The trial was also used to

investigate use of qPCR, an ELISA QuickStix™ test and mid-infrared spectroscopy to determine *B. cinerea* levels in juice samples. QPCR clearly showed that the fungicides reduced *B. cinerea* load while the ELISA tests were able to statistically separate the treatments. Spectroscopy and visual assessments were unable to statistically separate treatment effects, but there was a positive correlation between values measured using each method.

A whole-of-block experimental procedure was conducted during the 2008-2009 growing season to investigate spatial variation of BBR within a vineyard. Vine vigour, measured as plant cell density, was found to positively correlate with BBR severity. Disease increase was attributed to berry-to-berry spread, not that of new infections. The trial also investigated the effect of early (flowering) versus mid-season (PBC) spray application and both qPCR and visual assessments demonstrated that the PBC application was more effective than the flowering application.

This project clearly demonstrated that qPCR methods can complement traditional visual assessments in the quantification of BBR, and showed the usefulness for assessing management practices such as fungicide application and vineyard variation, and for determining vineyard factors that contribute to increased disease.

Publications

Conference Proceedings

Dunne KJ, Evans KJ, Bramley R (2010) Secondary spread may not be the main driver of within-season increase in the severity of botrytis bunch rot. 14th Australian Wine Industry Technical Conference, Adelaide, 2010.

Dunne KJ, Barry KM, Cadle-Davidson L, Evans KJ (2011) Quantification of *Botrytis cinerea* in grape berries with PCR – a research tool. Horticulture for the future Conference, 18-22 September 2011, Lorne, Victoria.

Evans KJ, Bramley RGV, Dunne KJ, Gobbett DL (2011) Whole-of-block experimentation enhances co-learning by researchers and farmers. Horticulture for the future Conference, 18-22 September, 2011, Lorne, Victoria.

Refereed Journal Articles

Bramley RGV, Evans KJ, Dunne KJ, Gobbett DL (2011) Spatial variation in 'reduced input' spray programs for powdery mildew and botrytis identified through whole-of-block experimentation. Australian Journal of Grape and Wine Research. V17 p341-350.

Saito S, Dunne KJ, Evans KJ, Barry K, Cadle-Davidson L, Wilcox W.F (2013) Optimisation of techniques for quantification of *Botrytis cinerea* in grape berries and receptacles by quantitative polymerase chain reaction. Australian Journal of Grape and Wine Research. V19 p68-73.

Workshop presentation

Dunne KJ (2010) Botrytis epidemiology and management in Tasmania. Presented at workshop WO7 Botrytis bunch rot in cool climate regions, convened by D. Mundy. Australian Wine Industry Technical Conference, Adelaide, July, 2010.

Table of Contents

List of Tables

List of Figures

Chapter One
Literature review: botrytis bunch rot of winegrapes

1.0. General introduction

This chapter provides information regarding the Tasmanian wine industry and the implications of Botrytis bunch rot in wine grapes. It also provides a review of the literature relevant to the thesis research and concludes by providing a rationale for the research conducted. A topic-specific review of the literature is presented in the introduction to each chapter detailing experimental results. The thesis concludes with a general discussion of the results and their significance for the wine-grape industry including recommendations for future research.

1.1. Introduction to the literature review

Botrytis bunch rot (BBR) of grapes (also known as grey mould), caused by the necrotrophic fungus *Botrytis cinerea*, is one of the most economically important diseases in both wine and table grapes worldwide. The estimated cost of BBR and other bunch rot diseases to the Australian wine industry is $52 million per annum (Scholefield and Morison 2010), affecting both yield and fruit/wine quality (Riley 2008; Scott *et al*. 2010).

During the last twelve years, the majority of research conducted in the Australian wine industry on BBR has focused on disease management strategies e.g. integrated pest management, spray timing, vine factors (Wicks 2002; Dry and Thomas 2003; Cole *et al*. 2004; Cole and Wiechel 2004; Cole 2005; Emmett *et al*. 2005; Braybrook 2007), and the implications and consequences of BBR at harvest and its effects on wine quality (Martin 2001; Emmett *et al*. 2004). Other areas of *B*. *cinerea* research

have included the biology of the fungus, infection pathways and its role in horticultural crops (Elmer and Michailides 2004; Evans 2008). The incidence and severity of BBR can be unpredictable as the pathogen has the ability to infect fruit at any stage during the season, but at the end of the season (later stages of ripening); the fruit may or may not exhibit the symptoms. *Botrytis cinerea* is highly adaptable to its environment and able to survive adverse conditions; examples include the ability of populations to develop resistance to fungicides, the ability to colonise other flowering plant species and a variety of horticultural crops (Pak and Wood 1995; Bézier *et al*. 2002; Elmer *et al*. 2005; Elmer and Reglinski 2006). Since *B*. *cinerea* is adapted to cool climates, the Tasmanian climate is ideal for pathogen growth and reproduction.

Botrytis bunch rot is an issue that the wine industry faces both nationally and internationally, due to the logistical, quality and management challenges it can cause for growers and the wineries. In order to implement optimal management practices for BBR within a vineyard, a greater understanding of the key events in the fungus' life cycle is needed as well as the many factors in the vineyard that affect the spread of the disease. *Botrytis cinerea* has the ability to infect fruit early in the season without symptom development. Once established, it has the ability to develop rapidly into BBR late in the season close to harvest, thus affecting grape quality. It is at this later stage that implementation of control measures is limited due to bunch closure preventing airflow between berries, fungicide restrictions such as maximum residue limits (MRLs) and withholding periods. With these limitations in mind, early detection of the disease prior to symptom expression could help in implementing control measures. The tools for early detection or monitoring of BBR are still in early stages of development. Techniques that are either available or still in development include ELISA (Ricker *et al*. 1991; Dewey *et al*. 2005), PCR-based techniques (Cadle-Davidson 2008; Celik *et al*. 2009; Diguta *et al*. 2010; Sanzani *et al*. 2012), loop-mediated isothermal amplification (Tomlinson *et al*. 2010) and spectroscopy-based methods (Cozzolino *et al*. 2003; Versari *et al*. 2008). By using molecular based tools and field trials in this project, the project aims to apply these methods to help in the understanding of botrytis epidemics.

1.2. The Tasmanian wine industry

Wine grapes have been grown in Tasmania since the 1820s on a very small scale until the 1970s when the industry began to grow rapidly to its current size (Coombe and Dry 1992a; Anonymous 2011a). Due to the cool climate, the regions are ideal for producing premium table and sparkling wines. In 2010-11, Tasmania's 1,392 ha of bearing vines produced a crush of 7, 791 tonnes (refer to Table 1.1) (Anonymous 2011b). The State contains seven wine regions: the Tamar Valley, East Coast, North East, Coal River Valley, Derwent Valley, the Huon/Channel region and the North West. All of the regions have similar climates that can be classed as cool maritime (Gladstones 1992, Anonymous 2011b). This maritime climate differentiates Tasmania's regions from other cool climate regions on the mainland of Australia (e.g. Clare Valley and Margaret River) and in Europe. The main feature of a maritime climate is that there are only slight temperature variations between summer and winter. The mean growing season temperature for the whole of Tasmania is 14.7 °C (ranging from 10.7 - 14.7 °C), with a mean January temperature of 16.8 °C and mean August temperature of 8.7 °C (refer to Table 1.1 for climate data). One limiting factor of the cooler climate is that vines tend to have more vegetative growth than fruit growth requiring implementation of canopy management practices (Coombe & Dry 1992b). The growing season temperatures of the wine growing regions of Tasmania are comparable to that of the great wine regions of Champagne and Burgundy, which allow for a slow ripening period (Sanderson 2012b). As a result, Tasmania is well suited to producing high quality sparkling wines and table wines.

The samples and data obtained in this PhD study were from the Coal River Valley region. This region has longer sunshine hours and lower rainfall per annum than the Clare River and Margaret River regions of mainland Australia (Gladstones 1992, Sanderson 2012b). The current mean January temperature (MJT) for the region is 17.0°C and mean August temperature (MAT) is 8.7°C, with a mean growing season temperature of 14.7°C (ranging from 9.3 - 20.2°C) (refer to Table 1.1). The main wine grape varieties grown in the Coal River Valley region are Chardonnay, Riesling, Sauvignon Blanc, Pinot Noir and Pinot Gris (Anonymous 2011b).

Table 1.1: List of Tasmanian wine regions with production figures and seasonal information for the 2011 season. Information sourced from Wine Tasmania (2011) for viticultural data. Weather data were calculated using available data from the Australian Bureau of Meteorology Website using data collected from a minimum of two weather stations (accessed 02/03/2012) and Wine Tasmania (Sanderson 2012a). Mean Growing Temperature (MGT) is shown and was calculated using the mean temperatures from October to April. Calculated mean January temperature (MJT), mean August temperatures (MAT) and mean annual rainfall are shown for each of the regions. The calculated heat degree days (HDD) are also shown during the growing season from October to April (Sanderson 2012a). Refer to Equation B1, Appendix B for the HDD equation.

Region	Bearing Area (ha)	Yield (tonne)	HDD	MGT (°C)	MJT (°C)	MAT (°C)	Mean Rainfall (mm)
Tamar Valley	473	2,649	1231	15.6	18.1	8.2	663.4
East Coast	265	1,480	1118	15.0	16.9	10.1	592.8
North East	251	1,402	1095	14.9	16.9	8.4	845.6
Coal River Valley	237	1,325	950	14.7	16.9	8.7	601.9
Derwent Valley	84	468	1101	15.1	16.8	8.8	566.3
Huon/Channel	70	390	828	13.8	15.8	7.9	709.5
North West	14	78	797	13.7	16.0	8.8	1069.9
Total	***1,392***	***7,791***	***1017***	***14.7***	***16.8***	***8.7***	***721.3***

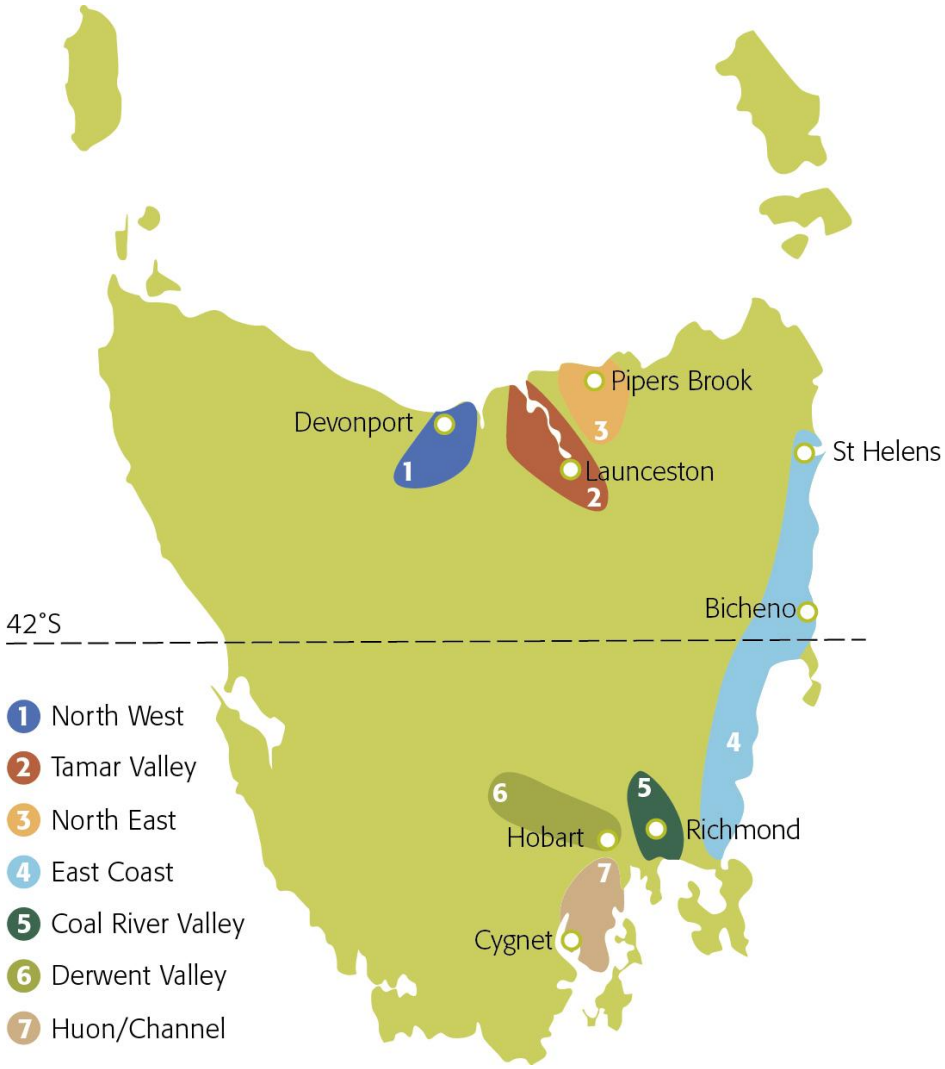

Figure 1.1: Map of the wine regions of Tasmania (Wine Tasmania, accessed 13/04/2014).

1.3. The development of BBR in grapes

Plant disease will not occur unless there is inoculum, favourable weather conditions and susceptible plant tissue. All three conditions must be present for disease to become established and expressed (Agrios 1997).

1.3.1. Sources of inoculum

The inoculum is the infective propagule that initiates disease in the host plant, in this case the spores (conidia) or sclerotia (producing conidia) of *B. cinerea* (Agrios 1997). Sources of inoculum include decaying plant tissue, which has been colonised by the fungus, which then produces conidia from mycelia or sclerotia bearing conidiophores (Agrios 1997). Once the tissue is colonised, it becomes a substrate for growth and reproduction of the pathogen. There are numerous sources of *B. cinerea* inoculum in the vineyard that may vary from region to region, but all can play a role in providing a source of spores for infection (Nair and Nadtotchei 1987; Elmer and Michailides 2004; Jaspers *et al*. 2013). In the Marlborough region of New Zealand, Jaspers *et al*. (2013) found that these sources included old rachides, tendrils, leaf petioles, and cane debris (pruning material from the previous season). In New South Wales, a survey of Hunter Valley vineyards found that sclerotia were the main source of inoculum for the following season (Balasubramaniam *et al*. 2000). Overall, the main sources of inoculum found in vineyards include dead rachides in the canopy or on the vineyard floor, herbicide-treated or senescing weeds in the inter-rows, tendrils, leaf petioles, cane debris, buds and canopy debris (flower caps, aborted berries, senescing plant tissue that is caught within clusters) (Holz *et al*. 2003; Elmer and Michailides 2004; Jaspers *et al*. 2013). During the main phase of disease development during fruit ripening, infected bunches situated near uninfected bunches also act as sources of inoculum for secondary infection.

1.3.2. Weather conditions

The ideal conditions for disease expression are presence of moisture in the form of rainfall, high relative humidity above 95% for a minimum of 15 hours, dew, or mist (Gubler *et al*. 1987; Nair *et al*. 1988; Thomas *et al*. 1988; Nicholas *et al*. 1994; Vail *et*

al. 1998). Once infection is established, the fungus does not require free moisture as it uses the moisture and sugars within the berry to continue to develop during ripening. The optimal temperature range for the fungus to colonise the plant host is 15 – 24 °C, but spores can germinate and survive anywhere from 1 to 30 °C (Coley-Smith *et al*. 1980; Hall and Emmett 2000).

1.3.3. Vine factors

Berry development is an important factor in BBR development. Unlike some other soft fruit pathogens, *B*. *cinerea* initially infects the grape berry during the period between flowering and early stages of development, but then goes into a quiescent/latent phase of no active growth (McClellan & Hewitt 1973, Nair *et al*. 1995, Keller *et al*. 2003, Elmer & Michailides 2007). This latency is thought to be induced by high amounts of antifungal phytoalexins in the young berries (Keller *et al*. 2003, Pezet *et al*. 2003). During *véraison* and ripening, the chemical composition of grape berries alters, as they grow and expand. There is an increase in sugars (measured as total soluble solids (brix), or baumé), increase in pH, a decrease in organic acids such as tartaric and malic acid, a decrease in antifungal phytoalexins, and changes in the concentration of tannins and phenolic compounds contained in seeds and skins (Mullins *et al*. 1992). The concentration of these chemicals is what may affect the ability of *B*. *cinerea* to infect or further colonise the grape berries (Mullins *et al*. 1992; Wolf *et al*. 1997; Gabler *et al*. 2003). Hill *et al*. (1981) observed a direct positive correlation between the extent of *B*. *cinerea* colonisation and berry sugar concentration, when berries were inoculated with *B*. *cinerea* conidia.

As berries ripen, deterioration of the waxy layer (waxy bloom) and the cuticle occurs, resulting in increased susceptibility of the berry to *B*. *cinerea* infection (Comménil *et al*. 1997). Studies have shown that adjuvants commonly used to help fungicides adhere to the plant's surface can alter the structure of the berry cuticle and break down some layers causing the berry to become susceptible to BBR (Rogiers *et al*. 2003; Rogiers *et al*. 2005; Elmer and Reglinski 2006). Rogiers *et al*. (2005), in laboratory studies, found that using the tested adjuvants with and without a fungicide application increased the incidence of *B*. *cinerea* infection compared with fungicide only. The study used adjuvants not registered for use on grapevine but noted that the

adjuvants were known to be used by growers. MacGregor *et al*. (2006), however, found no link between adjuvant use and increased disease severity in field trials conducted over a four-year period (2002 - 2005) in Australia and New Zealand. They did not investigate the effect of the adjuvants at the level of the grape berry cuticle.

Studies have also shown that sugars from within the berry are mobilised to the surface of the skin towards the later part of the ripening stage, thus stimulating *B*. *cinerea* to infect the berry (Kretschmer *et al*. 2007). Other factors that contribute to the spread of the disease within the grape bunch are the architecture and compactness (Vail *et al*. 1998; Dry and Thomas 2003). The more compact the bunch, the greater the pressure exerted on individual berries from adjacent berries, resulting in greater skin-to-skin contact area. This can cause the berries to split, resulting in new infection sites. Tight clusters can also result in the pedicel breaking under pressure, resulting in wounds and ideal sites for initial infection and a source of inoculum within the bunch (Nair *et al*. 1988; Vail *et al*. 1998; Gabler *et al*. 2003; Shavrukov *et al*. 2003). The physical make-up of berries in compact bunches is also altered, such that they have thinner skins and waxy layers. In addition, the bunches retain free moisture and the berries remain wet due to the limited airflow associated with tight bunches (Vail *et al*. 1998; Gabler *et al*. 2003). All these factors can promote both the spread and late season infection of the pathogen. Other host factors related to susceptibility (including canopy management, resistance of cultivars) will be explored in detail in section 1.7.

1.4. Impact of BBR on wine quality

Botrytis bunch rot creates numerous problems for the wine industry, beginning in the vineyard where severe infection leads to reduced yield and fruit quality, and can result in rejection of the fruit by the winery (Scott *et al*. 2010). Often when vineyard blocks have a high incidence of BBR but the severity is still within winery specifications, the fruit is harvested earlier than the intended date to prevent further disease development (Riley 2008). This may mean that the grapes are harvested before the physiological ripeness and desired flavours required for production of high quality wine have developed (Riley 2008). If fruit is accepted, but is outside of the contracting winery specifications, the grower faces the risk that the price offered would be lower than that of the current market price set by the company. This allows the company to

factor in the added costs at the receival end, due to the additional steps required during processing. Wineries require best-possible estimates of harvest dates to plan the logistics of receiving and processing fruit. An early harvest caused by BBR can adversely affect winery logistics and incur extra costs in organising labour and equipment (Godden 2000). These extra costs were not factored into the estimation of BBR cost to the Australian wine industry by Scholfield and Morison (2010) nor were the cost of remedial winemaking as discussed below.

The presence of BBR can also add unwanted costs to the winemaking process due to remedial measures required to minimise the impact of the fungus in the end wine (Godden 2000; Dumeau *et al*. 2004). During the winemaking process, *B*. *cinerea* can cause numerous taints resulting in a wine that smells and tastes of mould (phenol flavour/ aroma), overpowering the desired aromas present in the wine (Peynaud 1984). These taints arise from fungal enzymes such as laccase, which convert the grape berry sugars, fructose, and glucose, into glycerol and gluconic acid, promoting oxidation of the juice and leading to a wine that has characteristics of oxidative processes (Bulit and Dubos 1988; Mullins *et al*. 1992; Rankine 1998). Laccase is the main enzyme which, when present in the juice and wine, transfers oxygen molecules into colourless phenolic substrates, resulting in permanent browning of the juice, astringency, reduced flavour and off flavours (Peynaud 1984; Rankine 1998).

Often after crushing, the juice/must will only be in contact with the skins for a limited period in order to reduce further oxidation caused by the fungus during and after harvesting (Dumeau *et al*. 2004). Oxidation of the must has the potential to result in sub-optimal extraction of colour and flavour compounds (Dumeau *et al*. 2004). To minimise oxidation, higher additions of sulphur dioxide in the forms of potassium metabisulphite or sodium metabisulphite are required than that used for clean fruit (Dumeau *et al*. 2004). As *B*. *cinerea* releases the laccase enzyme in fruit, to deactivate the enzyme a pasteurisation step can be used (AWRI 2011; Smith 2011). This involves heating the wine quickly up to 60 °C for a short period and then rapidly cooling down using the specialised equipment (Smith 2011). However this can have added costly implications due to the equipment that is required and the additional treatment of the juice/wine. There is also the added risk of losing colour, flavour and aroma compounds.

Wine with negative characters caused by *B. cinerea* may be blended with other wine that is not suitable for the premium end of the market. This results in a lower-priced product such as bulk wine, or the wine is destroyed. During the winemaking process, it is impossible to eliminate the unwanted botrytis characteristics without also removing flavour, aroma, and colour compounds. Additional fining steps are also required to in order to remove as much of the 'off' flavour compounds in the wine before removing all the desired characteristics as well (Baldwin 2011; Steel *et al*. 2013). All of these steps will result in a wine of inferior quality than that which the grapes were originally destined, and will add significant costs to the production.

1.5. Symptom expression of *B. cinerea*

Colonisation of fruit by *B. cinerea* results in a pink/brown rot of berries in white grape varieties, which is hard to distinguish in red varieties (Bulit and Dubos 1988; Keller *et al*. 2003). After this initial symptom expression, under conducive climates, the characteristic grey fury growth that is the mycelia and conidia is expressed (Bulit and Dubos 1988; Keller *et al*. 2003; Jaspers *et al*. 2013) (Figure 1.2). This later symptom of the disease mainly occurs during the second stage of berry ripening or during post- harvest storage for table grapes and other fruits such as strawberries (Nicholas *et al*. 1994; Balasubramaniam *et al*. 2000; Williamson *et al*. 2007). *Botrytis cinerea* can also infect other plant organs including flowers, leaves (causing v-shaped lesions), shoots, stems and petioles (causing soft rot) (Nicholas *et al*. 1994; Hall and Emmett 2000; Williamson *et al*. 2007). The fungus has also reportedly caused green fruit rot in the Hunter Valley resulting from latent infections and weather conditions conducive to the development of the fungus (Nair and Parker 1985).

Figure 1.2: Cultivar Vignoles (French-American hybrid) growing in New York State showing symptoms of the pink brown rot and sporulation by *Botrytis cinerea*.

1.6. The infection pathways & life cycle

Botrytis cinerea has a wide host range of over 200 different crops world-wide, including grapes, kiwifruit, figs, stone fruit, ornamental flowers, strawberries and tomatoes (Cook *et al*. 2002; Elmer and Michailides 2004; Zhonghua and Michailides 2005; Williamson *et al*. 2007). In most of these crops, *B*. *cinerea* causes post-harvest storage rots, rather than pre-harvest rots, as is the case for wine grapes (Michailides and Elmer 2000; Williamson *et al*. 2007).

Botrytis cinerea is a necrotrophic pathogen, which can colonise dead or dying plant tissue and survive as mycelia or sclerotia on the tissue (Elmer and Michailides 2004; Williamson *et al*. 2007). A necrotrophic pathogen has the ability to either invade

dead tissues and/or actively promotes plant cell death using several different strategies (Van Kan 2006). These strategies include the release of toxic molecules in the form of enzymes and metabolites, induction of oxidative burst resulting in the bursting of the cell wall via the increase in the amount of certain molecules (e.g. H_2O_2), or via direct penetration with or without the use of the toxin to aid pathogen ingress (Van Kan 2006). Elmer and Michailides (2004) provided a detailed description of the possible pathways for *B. cinerea* to become established within the grape berry, as well as the spread of the disease (refer to Figure 1.3 for a simplified representation of the lifecycle). The two main infection pathways that lead to bunch rot are flower and fruit infection. When *B. cinerea* infects flowers, the conidia germinate and colonise cap scar tissue, but further growth of the fungus is arrested by antimicrobial metabolites (stilbenes) produced in the immature grape berries causing the fungus to go into a latent phase (Keller *et al*. 2003; Pezet *et al*. 2003). As the berries ripen, the level of antimicrobial metabolites declines and fungal growth resumes leading to colonisation of the berry (Dugan *et al*. 2002; Keller *et al*. 2003). After flowering and especially during fruit ripening, the pathogen can directly infect the fruit through wound sites (Wilcox 2002; Keller *et al*. 2003; Zitter 2005). These wound sites can be caused by infection by other pathogens such as the powdery mildew fungus (*Erysiphe necator*), physical damage, cracking of the cuticle arising from pressure either internally or from tight bunches, wind and wet conditions (Nair and Parker 1985; Nair *et al*. 1988; Gabler *et al*. 2003; Keller *et al*. 2003). Other factors that lead to greater levels of disease and spread include damage by pests such as light brown apple moth in Australia (Emmett *et al*. 1995) or bird damage (Bailey *et al*. 1997) and thrips in New Zealand (Schmidt 2007; Schmidt *et al*. 2007).

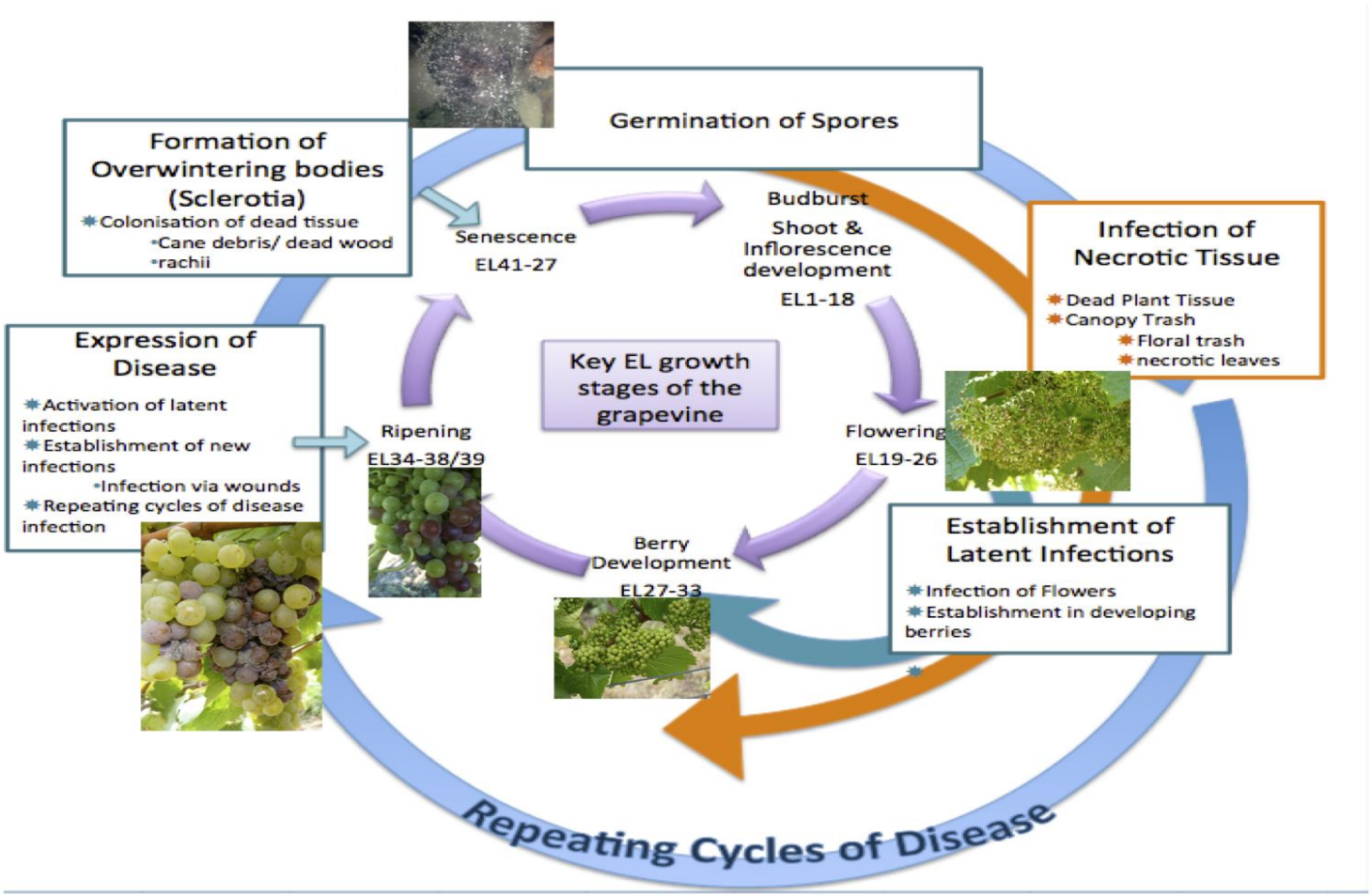

Figure 1.3: Simplified pictorial representation of the lifecycle of *B. cinerea*. Corresponding key grapevine growth stages during the disease development (purple). EL stage is based on that of the modified EL growth stage by Coombe (1995). Life cycle of *B. cinerea* is based on that of the figure presented by Elmer & Michailides (2004) and Pearson (1984). Orange arrows represents necrotic tissue pathway; Teal arrows represent latent infection; blue arrows show that it is a continuous cycle. Pictures used in this diagram were obtained during project.

1.8. Modelling & risk assessment for BBR

Many factors can contribute to the increased risk of BBR expressing at harvest. As previously stated the expression of BBR is highly weather dependant (Section 1.3.2). There are a number of others factors which an understanding is required in order determine the level of risk, which include the various vine and vineyard factors (Section 1.3.3).

Modelling and assessing risk of BBR for wine grapes involves the following: - regularly assessing weather conditions (forecasts for rainfall events), knowledge of the predisposition of the crop in question to develop BBR (e.g. sources of inoculum, previous season BBR expression levels, variety and clone), knowledge of *B. cinerea* infection pathways in conjunction with the growth stages of the grapevine. To date there have been a number of models developed to aid in the determination of the risk of BBR development using weather, inoculum levels (e.g. airborne spore count), and crop stage (Broome *et al*. 1995; Ellison et al 1998a; Ellison *et al*. 1998b; Beresford *et al*. 2006; Beresford and Hill 2008; Javier Rodriguez-Rajo *et al*. 2010; Leyronas and Nicot 2013). To date, the Australian wine industry has yet to adopt a model similar to that of the powdery mildew risk system that informs growers of days when there is a high risk of infection. These warnings are broadcasted in order to let growers know that they may need to spray to minimise/stop the infection based on a model developed in the USA (Gubler *et al*. 1999). Currently, there has been testing of a botrytis decision support model (BDSM), in which was initially developed by Plant & Food Research (New Zealand). The model has been calibrated using data from Australian trials in which this project was part of (GWRDC report UT0601 (Evans *et al*. 2010)). All of these models highlighted that weather plays an integral part in the expression of BBR.

For a BBR risk model to be used by industry, the model needs to have a knowledge of how epidemics can arise and develop within the crop, which to date most models have only relided on tracking of airborne conidia as a tool to measure the risk (Rodriguez-Rajo *et al*. 2010; Leyronas and Nicot 2013). Both of these studies noted that the key infection stages of *B. cinerea* are flowering onwards, however both studies did not investigate the effect of management practices or take not of the observed rot at

harvest, which is the important issue in which growers are aiming to minimise the outbreak at this time. The validation completed by Ellison *et al*. (1998a and 1998b) found that in comparison with the vineyard manager's normal practices and the expert opinon, the Ellison model recommend more fungicide application than either of these two practices, with the similar level of BBR severity achieved. They noted that the grower would develop their fungicide plan based on prior experience (knowledge of previous seasons), factoring in regional weather factors, which where this study was conducted (Riverina, NSW), BBR risk is relative low unless there is a rainfall event during ripening. Ellison *et al*. (1998b) noted these results where validating their model and mentioned that in order for a model to be successfully adopted by inudstry, it needs to be tested, across a larger number of vineyards and across different regions

Therefore, it could be said that with these previous studies in mind, an understanding of the dynamics of how the pathogen (*B*. *cinerea*) functions within a vineyard, vine, bunch and berry is key in order to develop a model in which can be reliably used to determin the need for fungicide application. It is already widely known that the key infection period for latent infections is the period between flowering and berry development, with very little options of control past this point. Knowing the risk and understanding how an epidemic can progress in a vineyard has the potential to enable a more strategic and cost effective approach in management than just relying traditionally spraying at each key berry stage staying within the withholding periods.

The main tool that growers have for managing bunch rot is the use of fungicides. However, reliance on fungicides for management of BBR increases the risk of fungicide resistance developing in the *B*. *cinerea* population. Environmental (weather, site, general climate) and vine factors will also determine disease risk and fungicide efficacy. Therefore, a more integrated approach is essential for effective disease management. Integrated disease management (IPM) is an approach that uses a combination of tactics to control diseases and pests within a vineyard, often resulting in reduced fungicide inputs (Bernard *et al*. 2007). These tactics combine the most suitable chemical, biological, and cultural methods.

1.8. Vineyard management of BBR

1.8.1. Chemical control

The purpose of chemical control of a plant disease is to prevent or limit symptoms developing in the host crop (Agrios 1997). Contact fungicides act on the surface of plant tissues and are therefore typically protectant in activity (examples of fungicide groups include 2 & M4 (refer to Table 1.2)). Systemic products penetrate the cuticle and may be translocated within the plant, therefore some can act curatively after infection has occurred (usually up to 3-5 days following infection, known as the "kick back" period) (fungicide groups include 9, 9+12, 17 (refer to Table 1.2)). There are also fungicides that have both a contact and systemic mode of action, which tend to be fungicides that have dual chemistries (group M4 +4, (refer to Table 1.2)). In order to obtain greatest efficacy, contact and most systemic fungicides need to be applied prior to pathogen establishment (Agrios 1997, Evans 2008; Jacometti *et al*. 2010). In order for any fungicide to work, but particularly for contact fungicides, the active chemical must be able to adhere to the plant surface for long periods.

In the case of BBR control, contact fungicide applications may be applied prior to a rain event to limit establishment during berry development and early stages of ripening. Use of surfactants reduce the loss of the fungicide during rain events, enabling greater protection from the fungicide (Agrios, 1997).

Botrytis cinerea is known to develop resistance to fungicides with the active ingredients of benzimidazoles (no longer registered for use in Australia), dicarboximide, anilinopyrimidine, fenhexamid and fludioxonil (Wicks and Hall 2003; Korelev *et al*. 2011). Using an IPM approach can delay or prevent the development of resistant populations (Pak and Wood 1995). The ability of *B*. *cinerea* to survive as mycelium, conidia or sclerotia in dead tissue for long periods, its varied infection pathways and, as previously stated, its numerous sources of inoculum all contribute to its ability to develop fungicide resistance (Williamson *et al*. 2007).

The Australian Wine Research Institute (AWRI) publishes a booklet annually that contains recommendations on fungicides available for use by growers. The information is updated regularly to inform industry of changes and is available online (Essling and Longbottom 2013). Fungicides are grouped according to their active ingredient and each group is assigned a number (refer to Table 1.2 for fungicides registered for use in Australian viticulture). When developing a vineyard spray program, the recommended resistance management strategies should be considered along with the industry guidelines and contracting wineries (CropLife 2011; Essling and Longbottom 2013). Consecutive sprays of a particular fungicide should be avoided within both season and following seasons to prevent resistance from building up (Essling and Longbottom 2013). Disease development late in the season is difficult to control as most fungicides registered in Australia cannot be used close to harvest due to withholding periods imposed for wine exports. Withholding periods are set to ensure that fungicide residues that may be detected in wine are below a maximum residue limit (MRL). These restrictions have been put in place to protect consumers from any potential harm that may occur. To minimise the MRL in the end product, the industry has set guidelines for when certain fungicides can be sprayed and how many days prior to harvest (Essling and Longbottom 2013). The registered fungicide Filan® (active constituent boscalid), for example is no longer recommended for use on grapes destined for export wine as the manufacturer cannot define a time in the growth stage it can be applied where there is no MRL at harvest (Essling and Longbottom 2013).

Table 1.2: Fungicide groups available for the control of *B. cinerea* in Australian Viticulture. Examples of registered Fungicides used for the management of Botrytis bunch Rot in Australian Viticulture. Data sourced from Essling & Longbottom (2013) and CropLife (2011). CF- capfall; HA- harvest; d- day; EL (modified EL system) for vine stage according to Coombe (1995).

Fung Group	Chemical Group	Active Constituents	Registered Products	Resistance Found [a]	Restrictions
2	Dicarboximides	iprodione procymidone	Rovral Aquaflo® Chief Aquaflo®	Yes	Use no later than 7d before HA (EL35-38)
9	Anilinopyrimidine	pyrimethanil	Scala 400 SC® Pyrus 400 SC®	Yes	Use no later than 80% CF (EL25)
11	Methoxy acrylate	azoxystrobin	Amistar 250 SC® Mirado 250 SC®	No	
M5	Chloronitriles	chlorothalonil	Bravo 720® Whack 900 WG®	No	
17	Hydroxyanilide	fenhexamid	Teldor 500SC®	Yes	Use no later than 80% CF (EL25)
M4	Phthalimide	captan	Captan 900 WG® Merpan®	No	Use no later than 30d before harvest (approx EL33 latest)
U1	Potassium Salts of fatty acids		Ecoprotector®	No	Use no later than 14d before HA (EL35-38)
M+M	Hydrogen peroxide + peroxyacetic acid		Peratec® Peroxy Treat®	No	Use no later than 7d before HA Used for suppression (EL35-38)
9 +12	cyprodinil + fludioxonil		Switch®	Yes	Use no later than E-L 31 (Before PBC) and not within 60d harvest Do not use consecutively during the season (El32)
M4 +4	captan + metalaxyl		Duplex WG®	No	Use no later than 30d before harvest (approx EL33 latest)

[a] Refer to agrochemical guidelines for resistance management strategy.

The architecture of ripe grape bunches can hamper fungicide penetration as the berries touch each other, especially in tight or closed bunches (Dry and Thomas 2003). During fruit development, the bunch goes through a phase that is referred to as pre bunch closure (PBC), which occurs prior to the ripening period. This is characterised as the period immediately before the stage when berries are starting to touch each other within the cluster (Coombe & Dry 1992a; Coombe 1995). Tight bunches prevent fungicide penetration into the centre of the bunch, where *B. cinerea* could be established in a berry as a latent infection or in trapped flowering trash (Dry and Thomas 2003; Emmett *et al*. 2005). Wounds are also common where berries rub against each other or insects burrow into the bunch (Dry and Thomas 2003; Emmett *et al*. 2005).

Some fungicides require the addition of an adjuvant to maximise the efficacy of spray application. Adjuvants act as "spreaders" to aid spray coverage, including spray droplet contact, by reducing droplet surface tension and optimising retention of the fungicide on the plant tissue surface (e.g. bunches, leaves) (MacGregor *et al*. 2006). However, as mentioned in Section 1.3, adjuvants were shown to degrade the waxy cuticle layer of the berry, a natural defence barrier, in laboratory experiments (Rogiers *et al*. 2005), although this has not yet been demonstrated in the field situation. Adjuvants have also been reported to increase the retention of floral trash within the bunch, providing a potential inoculum source during the season as fungicide efficacy reduces (Wolf *et al*. 1997; Jaspers *et al*. 2013).

As previously mentioned, spray timing is an important factor of chemical control. It is important to maximise the effectiveness of the fungicide, yet meet winery/industry requirements concerning MRL (Wicks and Hall 2003). Restrictions on fungicide timing are related to grapevine growth stages, so that specific chemicals are not used after a certain point to ensure restrictions are met (Essling and Longbottom 2013) (Table 1.2). The crucial time of spray application for *B. cinerea* control is during the grapevine growth stages of flowering, PBC and *véraison*, which also takes into consideration industry restrictions (Coombe and Dry 1992b; Edwards *et al*. 2009; Evans 2010). Accurate identification of vine growth stages is important in order to apply the fungicide at the crucial time for optimal control and adhering to industry regulation (Evans and Gadoury 2008). To control flowering infection of *B. cinerea*,

the optimum spray period is around 80% capfall in order to prevent infection of the necrotic tissue around the inflorescence (cap scar) and in trapped floral trash (Keller *et al*. 2003; Evans and Gadoury 2008). Defining 80% capfall accurately requires regular monitoring of selected vines within a block to account for variation that may occur. Evans & Gadoury (2008) showed that flowering is not a uniform event and that within one cultivar there will be a high amount of variation, and the length of flowering is climate, region and vineyard dependent.

Over the last 12 years, there have been several investigations into spray timing, which focused on botrytis management involving targeted application based on disease risk (Agnew *et al*. 2004, Edwards *et al*. 2009). Investigations have included the comparison between a typical full spray program (includes early, mid and late season sprays), early season, mid and late season only applications (Edwards *et al*. 2009). Agnew *et al*. (2004) found that a targeted approach using the Bacchus model as a tool to assess *B*. *cinerea* risk reduced fungicide inputs, with fungicides applied only if vines were at key susceptible growth stages during favourable weather conditions. Edwards *et al*. (2009) found that there was no clear answer to which spray-timing events achieved the best BBR control. Some trials showed that the early season sprays worked better while others showed that the mid-season sprays were more effective and late-season sprays provided no better control than early season applications. Both studies highlighted that regions will differ in their response to different spray timings; however, results of spray timing trials conducted over numerous seasons to cover the range and variety of seasonal conditions might help evaluate the optimal time to apply fungicides in most seasons. The results might also give clues as to the main infection pathway for *B*. *cinerea* at a particular site. However, if the weather is particularly conducive to BBR in the lead up to harvest, the effectiveness of a spray program may be compromised (Riley 2008).

1.8.2. Biological & alternative control measures

Recently there has been a shift towards using other methods to control BBR rather than relying on fungicides for disease management (Reglinski *et al*. 2005; Elmer and Reglinski 2006; Beresford 2010; Jacometti *et al*. 2010). The wine industry is

constantly adapting to the expectations of its market and its environmental footprint, which is leading to practices that include less pesticide inputs, for both resistance management and environmental sustainability. Elmer and Reglinski (2006) and Jacometti *et al*. (2010) have reviewed alternatives to fungicides for the control of BBR in New Zealand due to the greater uptake by the wine industry there.

Biological control is the use of biological agents as an alternative or in an integrated approach to reduce fungicide input, where there is said to be three main types: classical, conservation and inundative (Jacometti *et al*. 2010). The classical and inundative biological control measures are the main types used in viticulture (Jacometti *et al*. 2010). The purpose of a classical control method is to permanently introduce a biological control agent into the environment for long-term control (Eilenberg *et al*. 2001; Jacometti *et al*. 2010). This type of control for *B*. *cinerea* is yet to be fully investigated (Jacometti *et al*. 2010). Inundative control measures involve the mass release of living organisms on more than one occasion to control or supress the target pest (Eilenberg *et al*. 2001; Schiler *et al*. 2002; Segarra *et al*. 2007; Jacometti *et al*. 2010).

The mechanisms in which biological control agents (BCAs) work include direct parasitism, competition, induction of the plant's natural disease resistance, production of inhibitory compounds, or modification of the environment on the plant surface (Elmer and Reglinski 2006; Segarra *et al*. 2007; Jacometti *et al*. 2010). The BCAs that have been studied for controlling *B*. *cinerea* diseases of various plant hosts include fungal organisms such as *Trichoderma* species (e.g. *T*. *harzianum*) (Elmer & Reglinski 2006; Segarra *et al*. 2007), *Ulocladium atrum* (Kessel *et al*. 2002), *Ulocladium oudemansii* (Elmer *et al*. 2005), yeast (Cook 2002; Rabosto *et al*. 2006) and bacteria strains (Rabosto *et al*. 2006; Magnin-Robert *et al*. 2007). *Trichoderma* species have been widely researched for ability to suppress plant fungal pathogens in grapes via antagonism (Elmer and Reglinski 2006; Vinale *et al*. 2008). They have been widely studied for the control of *B*. *cinerea* in grapes and other crops and a number of commercial products have been produced, the first being Trichodex using *T*. *harzianum* (Elmer and Reglinski 2006). The fungus suppresses *B*. *cinerea* by colonising senescent tissue and has the ability to colonise green tissue without causing harm, becoming established before the pathogen. The fungus *U*. *oudemansii* is also a

commercially available product that works via antagonism, competing and suppressing *B*. *cinerea* during colonisation of necrotic tissue found in bunches and canopies. A study by Elmer *et al*. (2005) over a number of seasons found that if the product was applied continually during the season the control could be as effective as fungicides. However, the study did note that late season control was not as effective as a fungicide application, when weather conditions were conducive to BBR. The study at the time also highlighted the need for further work into biological control options during *véraison* where the BCA agent tested was not as effective compared to its use earlier in grape berry development.

There have also been investigations into the efficacy of compost extracts in the control of BBR and grey mould in grapes and other crops, (Elad and Shtienberg 1994; Evans *et al*. 2013). These methods work by the mode of suppression, antagonism or via induced natural resistance of the plant (Elad and Shtienberg 1994; Pal and McSpadden Gardener 2006). There has also been investigation into the role of soil microbiology in reducing inoculum load of *B*. *cinerea* in vineyards (the role of mulches is discussed in a later section).

The limiting factors in the use of BCAs are that the organisms will have optimal environmental conditions that include temperature, humidity, and sunlight exposure for optimal microbial growth to control *B*. *cinerea* (Williamson *et al*. 2007). These BCAs are also subject to other organisms that may be naturally present in the plant as well as fungicides/pesticides that are used which could affect the efficacy of the BCA (Williamson *et al*. 2007). Therefore, BCAs will only give limited control by themselves, which may result in a higher level of BBR being present in crops than desired.

Another alternative to traditional control measures, which has been investigated, is in the area of botanical extracts where volatile compounds that are extracted from other plants have been used to suppress *B*. *cinerea* (Kulakiotu *et al*. 2004). Kulakiotu *et al*. (2004) investigated the potential use of volatile compounds extracted from *V*. *labrusca* cultivar Isabella in suppressing growth of *B*. *cinerea* cultures, as opposed to a highly susceptible *V*. *vinifera* cultivar Roditis. The study found that the volatiles released by the cv. Isabella inhibited the growth of the fungus, and highlighted the

potential use of these natural occurring fumigants as natural alternatives to suppress *B. cinerea*. There have also been investigations into the use of chemical additions to plants that stimulate the plant's resistance to diseases (Reglinski and Kingston 2001). These elicitors include calcium (further discussed in vine nutrition), chitosan, and naturally derived elicitors or those manufactured from plant/ microbe extracts (Reglinski and Kingston 2001).

1.8.3. Cultural methods

Cultural methods such as pruning regimes, canopy management, soil moisture monitoring, vine nutrition, inoculum removal (vine prunings, canopy trash) and choice of planting material with less pest susceptibility (disease and insects) can all help to manage disease outbreaks and severity (English *et al*. 1989; Nicholas *et al*. 1994).

A microclimate is created by the vine canopy in the region immediately surrounding the fruit and has the most direct influence on the pathogen and disease development. The vine canopy affects several factors including airflow, relative humidity, spray and light penetration (Gubler *et al*. 1987; English *et al*. 1989; Smart and Robinson 1991; Emmett *et al*. 1995). Dense canopies promote disease by providing a humid environment with free moisture and limiting fungicide penetration resulting in poor protection of the fruit. The choice of both the trellis type and row orientation when establishing a vineyard determines the vigour and eventually the structure of the canopy, which can potentially affect the development of fungal diseases. Growers can control vigour when establishing a vineyard by the use of rootstocks, which can minimise the vegetative growth and reduce canopy density (Elmer and Michailides 2004; Jacometti *et al*. 2010). The choice of pruning regime adopted can have a direct effect on crop load and canopy microclimate, which can affect BBR development (Smart and Robinson 1991). Research has found that there are a number of vine management options available to limit disease risk (Gubler *et al*. 1987; English *et al*. 1989; Coombe and Dry 1992; Mullins *et al*. 1992; Emmett *et al*. 1995). Both Gubler *et al*. (1987) and English *et al*. (1989) found that the removal of leaves around the grape bunches led to a significant reduction in the amount of BBR in the control

treatments at the end of the season. Gubler et al (1987) investigated the effect of several canopy manipulation techniques with and without fungicide (flowering, PBC, flowering + PBC) on the incidence and severity of BBR. The treatments were no canopy management, hedging, leaf plucking, a movable wire system and shoot removal, all of which were tested in treatments with or without fungicide over two years (1984 and 1985). Overall, the study found that leaf plucking was the optimal method for canopy manipulation in decreasing the incidence and severity of BBR and aided in fungicide control compared to the control treatments. The English *et al*. (1989) study investigated leaf removal and the effect it had on the canopy microclimate. They observed that the microclimate differences in humidity and temperature were not significant, but the wind speed was greater in the treatment with leaf removal than the treatment without. A recent study conducted by Edwards *et al*. (2009) also found that leaf plucking significantly decreased botrytis severity in trials conducted in New Zealand and Victoria. Airflow is also affected by row orientation, highlighting the important role of vineyard establishment (Smart and Robinson 1991; Mullins *et al*. 1992). Bunch thinning can have both a direct and indirect impact on botrytis severity, and in severe infections bunches will be dropped prior to harvest so that only the clean or least infected fruit are harvested (Barbetti 1980). High crop loads can lead to a more crowded bunch zone, resulting in a microclimate suitable for BBR (Guilbaud-Oulton 2000). Crop load is also determined by pruning level, which will also affect airflow in a canopy, both of which can affect BBR severity (Smart and Robinson 1991; Mullins *et al*. 1992; Nicholas *et al*. 1994). In seasons where there is high disease level at harvest, often bunches are removed prior to harvesting in order to minimise the amount of diseased fruit harvested, or clean fruit is selectively hand harvested where mechanical harvesting would normally have been used (Riley 2008).

In managing BBR, the soil/ground environment is also a factor. Trials have been completed on the role of soil moisture in promoting the severity of BBR (Wilcox *et al*. 2006). Wilcox *et al*. (2006) conducted a study using potted grape vines and found that the vines subjected to higher soil moisture content during ripening resulted in fruit with a higher severity of BBR. The application of mulches to the soil below the vine has been found to reduce both the inoculum and the sources of inoculum in vineyards for *B. cinerea* (Jacometti *et al*. 2007). Jacometti *et al*. (2007) investigated the effect of four mulches placed under the vines (anaerobically fermented grape

marc, aerobically fermented grape marc, inter-row grass clippings and shredded office paper) on the level of *B. cinerea* inoculum in comparison with bare earth over two years. The studies found that all four mulch treatments resulted in reduced amount of inoculum on the vineyard floor, which inversely correlated with a higher amount of vine debris decomposition and increased soil biological activity. The mulches also resulted in significantly less BBR than the non-mulch control treatment (Jacometti *et al*. 2007).

Vine nutrition is another aspect of vineyard management, which may have both direct and indirect consequences on disease severity. Nitrogen is an important nutrient for ensuring healthy vines and at harvest, low levels of Yeast Available Nitrogen (YAN) can have a negative impact on fermentation (Bell and Henschke 2005). There are conflicting reports on the role of nitrogen in BBR and recent studies have shown that there appears to be no direct correlation between nitrogen levels and BBR severity (Mundy and Beresford 2007). However, there may be indirect effects, as too much nitrogen can lead to excessive vegetative vigour resulting in large canopies. This increases the risk of BBR by affecting fungicide penetration and promoting a microclimate conducive to the disease (Smart and Robinson 1991; Bell and Henschke 2005). Calcium has been found to play an important role in BBR susceptibility. Studies have shown that calcium levels in grape berry cell walls play a vital role in the reduction of botrytis infection in grapes. Calcium is thought to chelate the pectic substances that are emitted by the fungus as it tries to infect the cell walls (Chardonnet *et al*. 1997; Winter and Nicol 1998). Winter & Nicol (1998) found that applying calcium directly on the vines during early stages of berry development helped to reduce BBR severity. Chardonnet *et al*. (1997), via an inoculation study, applied calcium on harvested berries with results suggesting that calcium did not influence BBR infection.

There is evidence that certain cultivars and clones of grapevines are less susceptible to BBR than others (Elmer and Michailides 2004). The most susceptible cultivars include Pinot Noir, Chardonnay, Sauvignon Blanc and Riesling (Nair and Parker 1985; Vail *et al*. 1998; Howell 2011). A study conducted by Gabler *et al*. (2003) on post-harvest susceptibility in table grapes found that resistance was positively correlated with the number and thickness of epidermal and hyperdermal cells, cuticle

and wax content, while there was an inverse relationship with pore number. In disease-prone situations, growers can choose cultivars accordingly and implement management practices to minimise susceptibility. During the initial stages of vineyard development there is the opportunity to select varieties, clones, and rootstocks (for vine vigour control) which may potentially help to reduce BBR risk. Vine vigour is also determined by variety, clone and rootstock, and vigorous vines can produce dense canopies resulting in a microclimate suited to the development of *B. cinerea* (Gubler *et al*. 1987; Fermaud *et al*. 2007; Valdés-Goméz *et al*. 2008; Jacometti *et al*. 2010). It is at this stage that informed decisions relating to disease susceptibility and climate factors can be considered. In high-risk regions, earlier ripening varieties may be chosen to minimise risk (Riley 2008).

Damage to berries can lead to increased risk of *B. cinerea* infection and spread later in the season. Damage to berries can occur via splitting due to physiological factors, or physical damage via insects, birds or mechanical/environmental agents. Emmett *et al*. (2005) found that controlling Light Brown Apple Moth (LBAM) led to a reduction in BBR incidence and severity. LBAM is known to be a vector of the disease, and it can cause damage to bunches early in the season during flowering until harvest. In New Zealand, thrips (*Thrips obscuratus*) have been found to vector the fungus (Schmidt 2007). Research conducted under controlled glasshouse conditions tracked the spread and infection of a mutant strain of *B. cinerea* during flowering and assessed the damage at harvest (Schmidt 2007). The study found that the insect led to an increased incidence of *B. cinerea* in white varieties (Riesling and Sauvignon Blanc) but not in Pinot noir, as well as resulting in lower berry numbers in the bunch (Schmidt 2007). The resulting increase in BBR, according to Schmidt (2007), was possibly due to *B. cinerea* entering the wounds created by the insect.

1.9. Precision Viticulture, spatial variability & disease

There is often considerable variability in topography and soil type across a vineyard block. Understanding how this variability affects disease outbreaks and severity within a vineyard is important for disease management. Precision Viticulture (PV) is a form of precision agriculture that allows growers to understand and manage the variability that can occur in growing grapes (Bramley and Hamilton 2004). PV-based trials take into consideration the variation that occurs across the farm (e.g. soil, water, crop vigour and microclimate) compared to the commonly used 'small plot' trials that may be set-up in a small section of the farm (Panten *et al*. 2010). PV research to date has focused on factors affecting grape quality (Bramley 2006; Panten *et al*. 2010) and had only limited use in the study of grapevine diseases and pests such as powdery mildew (Bramley *et al*. 2011a; Bramley *et al*. 2007) downy mildew (Stoll *et al*. 2008) and phylloxera (Bruce *et al*. 2009).

Although there has been limited use of PV in epidemiological studies of grape diseases and pests, it has been widely used in Precision Agriculture (PA). Optical remote sensing is a precision tool, which can aid detection of stress, including disease. This may be via 1) selected wavelengths developed for particular diseases or pests and crops, for example necrosis in eucalypts (Barry *et al*. 2011), leaf spot in apples (Delalieux *et al*. 2007) or pest damage in grapevine (Blanchfield *et al*. 2006), or 2) general assessments of leaf biomass via indices such as the normalised difference vegetation index (NDVI) which is used widely in precision agriculture (Haboudane *et al*. 2004; Bruce *et al*. 2009). If environmental factors or nutrient deficiency can be ruled out, the stress may be due to fungal infections (Jacobi and Kühbauch 2005; Tartachnyk *et al*. 2005). These images are then used to determine if sections of the crop need extra control in the affected areas. Remote sensing detection of BBR would be possible after symptom development, due to the colour change of infected berries, however quantitative accuracy may be reduced by the presence of foliage. Rather than the use of NDVI to detect low leaf area as a symptom of stress, it is postulated that high NDVI may be associated with greater BBR risk. By combining aerial imagery and soil surveys, Bruce *et al*. (2009) were able to identify potential

phylloxera infestation risk sites, which may not have been readily identified at ground level in low vigour blocks due to potential water and nutrient deficiency that may have been observed.

Bramley *et al*. (2007, 2011a) investigated the use of a whole of block experimental design for studying powdery mildew (caused by *Erysiphe necator*), focusing on two organic spray programs. To develop the spatial maps, data was collated from 230 target vines whose positions were determined by a global positioning system (GPS). The study was able to determine the effectiveness of the spray program in a commercial block, with sprays applied using available vineyard spray rigs and tractors. The results were able to take into consideration the role of variation that will occur in any block, due to vine vigour/soil/microclimate, while using the more traditional 'small plot' these factors would not be considered. The study found that a sulphur-based spray program resulted overall in significantly less powdery mildew development over time and across the majority of the block than the treatment using the same program, but with one fewer application of sulphur. The trial also showed that efficacy of a fungicide program will vary across a block, which may result from other vineyard factors that include vine vigour, soil type and variation in spray penetration (spray output variation and/or canopy density). In comparing the two fungicide treatments, the results showed that the degree of significant differences varied, with some sections showing no significant difference between the two treatments. The results of the experiment found that the slope of the block was potentially a contributing factor to the observed incidence and severity of powdery mildew. The study surmised that where the greater incidence and severity of powdery and the decrease in significant differences between treatments was observed, the causing factor may have been due to the particular section of block being subjected to the longer periods of shade during the day, due the position of the block in the vineyard (Bramley *et al*. 2011).

By utilising a whole block approach, it allows the researcher to fully understand the dynamics of a disease in a 'real situation' as the approach requires the use of whole rows repeated across the block (e.g. two treatments in lots of 6 rows across a block of 60 rows). This contrasts with the more commonly used 'small plot' layout where each treatment is a single or small number of panels and the trial is situated in one

section of the block (Panten *et al*. 2010; Bramley *et al*. 2011). The limitation of a small plot experiment is that the treatment differences observed may differ in another section of the block if such experiment was conducted concurrently. This could mean that in one section, where a small plot experiment is conducted, significant differences between treatments may be obtained, but if repeated in another section of the block there may be no treatment significance. With this in mind, the use of the 'whole of block' experimental procedure is needed in order to understand the epidemiological factors that contribute to *B*. *cinerea* development within a vineyard block, to account for variation that will occur in normal commercial practices.

1.10. Detection methods for the study of *B*. *cinerea*

Reliable detection methods of pathogens in plant or fruit samples are essential in order to study disease progress and the effects of treatments, with the potential for future adoption by industry, as methods become more streamlined. There are several detection methods that have been commonly employed in the detection of *B*. *cinerea* including visual observation in field and/or via moist incubation of samples, Enzyme-Linked Immuno Sorbent Assay (ELISA) tests and real-time quantitative polymerase chain reaction (qPCR), and recent investigations into the potential of spectroscopy (Scott *et al*. 2010).

Moist incubation is a technique used to assess pathogen presence in samples of plant tissue that may not necessarily be showing symptoms. Generally, for assessing *B*. *cinerea* latent infection, the method involves taking berry/bunch samples at the pea-size/ PBC growth stage (Holz *et al*. 2003). The berry tissue is then surfaced sterilised and washed before or after being subjected to a freezing or herbicide-treatment (paraquat) step (this is followed by another washing step) (Holz *et al*. 2003, Cadle-Davidson 2008). Freezing or treating with paraquat breaks down the natural defences within the developing green berry, which are thought to prevent the growth of *B*. *cinerea*. After moist incubation, the tissue samples are then assessed for *B*. *cinerea* sporulation (Holz *et al*. 2003; Cadle-Davidson 2008). Holz *et al*. (2003) used several moist incubation methods to determine where the fungus originates from within the berry and found that latent infection is towards the pedicel and base of the berry.

Cadle-Davidson (2008) investigated the use of moist incubation and the use of real-time quantitative PCR (discussed in the next section) for the detection of latent infections in grapes. The study used a modified protocol based on that of Holz *et al*. (2003) for the incubation technique and used a new qPCR assay to compare results. Although both methods were able to detect latent infections, there was little correlation between the data sets as the study showed that the qPCR assay was able to detect infections when the moist incubation assay did not. In addition, the qPCR method was able to accurately quantify the relative amount of *B*. *cinerea* colonisation within the berry sample as the season progressed. However, there were instances where the qPCR was unable to detect *B*. *cinerea* in the berry at earlier growth stages whereas the incubation technique gave a positive result. This may have been due to the incubation technique allowing *B*. *cinerea* to grow within the berry and then sporulate overtime as opposed to the qPCR, which measures the actual amount of *B*. *cinerea* DNA present in the berry at that time point. The study also highlighted that further investigation was needed to test the limit and reliability of the qPCR in monitoring latent infection.

Even though moist incubation techniques provide a cheap alternative to new molecular based methods, there are some limitations of the technique. One factor to consider is time; it may take up to two weeks for the plant tissue to exhibit symptoms, particularly if the incubating techniques are not optimal (McCartney *et al*. 2003). Another factor to consider is that the person assessing the samples needs to be trained in identifying the fungal/bacterial/yeast pathogens that may egress from the incubating tissue (McCartney *et al*. 2003). In applying the assay to the detection of *B*. *cinerea*, there is also the issue of other yeasts/ bacteria/ fungal pathogens that may have been present in the berry that may become expressed during incubation and potentially inhibit *B*. *cinerea* growth. Adopting the right moist incubation technique to suit both plant and target pathogen is important as the incorrect procedure could result in false negative results being recorded.

The use of molecular-based techniques in the study of *B*. *cinerea* has mainly concentrated on population diversity studies, isolation of specific genes relating to pathogenicity, and identification of *Botrytis* species in diseased plant samples (Thompson and Latorre 1999; Zheng *et al*. 2000; Moyano *et al*. 2003; Schena *et al*.

2004). Recently there have been advances in molecular methods for quantifying *B. cinerea* in grape tissue, specifically ELISA assays and quantitative PCR (qPCR or real-time PCR) (Rigotti *et al*. 2002; Obanor *et al*. 2004; Ward *et al*. 2004; Dewey *et al*. 2005; Suarez *et al*. 2005). The main objective has been to find an efficient and reliable method to detect non-visible, latent infections.

1.10.1. ELISA (Enzyme-linked immunosorbent assay)

Enzyme-linked immunosorbent assays work on the basis of recognition, where for a positive result the target-specific antibody must recognise the corresponding antigen in the sample (Boonham *et al*. 2008). There are three types of antibodies, which include polyclonal (PAbs), monoclonal and phage display, all of which differ in their method of production (Ward *et al*. 2004). PAbs are produced via the injection of target pathogen extracts into animals (e.g. rabbit); after a period, a blood sample is taken and serum (antibodies) is removed from the clotted blood (Ward *et al*. 2004). This type of antibody is known to be used in plant pathogen detection, but has been found not to be very specific due to the production method resulting in limited amounts and batch variation (Ward *et al*. 2004).

The production of monoclonal antibodies involves fusing lymphocytes taken from inoculated animal host myeloma cells that have been cultured resulting in hybrid cells (Ward *et al*. 2004). Each hybrid cell contains a single monoclonal antibody, which is then propagated using cell culture media. Unlike the PAbs, monoclonal antibodies are more specific and homogeneous due to the production method (Ward *et al*. 2004). However, the production method is more time consuming and costly than the other two ELISA types (Ward *et al*. 2004). Phage display antibodies are produced via the use of the polymerase chain reaction (PCR) (read further on for a detailed description of PCR) (Ward *et al*. 2004). The method uses libraries of functional fragments for the antibody molecules and makes copies via the PCR technique. This type of antibody is highly specific and is commonly used in the production of immuno-diagnostic assays for plants (Ward *et al*. 2004). The advantages of this type of antibody production

method are that it is relatively cheap, less time consuming and does not require animal hosts for production (Ward *et al*. 2004).

ELISA tests have been readily adopted in plant pathology research and related industries (Ward *et al*. 2004; Boonham *et al*. 2008). They are often quick and easy to perform, cheap and depending on the assay type, may not need specialised equipment unlike PCR based assays (Ward *et al*. 2004; Boonham *et al*. 2008). Traditionally ELISA has been used mainly to detect plant viruses, however in recent times there has been application of the technique for fungal pathogens. Developing the assay for fungal detection is more difficult due to the complex DNA structure, as viruses only consist of a small genome of either single stranded DNA or RNA while fungi consist of a larger genome of double stranded DNA (Glick and Pasternak 1998; Tortora *et al*. 2001). Due to this factor, the reliability of ELISA tests for use in fungal infections may be limited (Ward *et al*. 2004; Boonham *et al*. 2008).

ELISA methodologies have been developed and produced as commercial kits for detection of *B*. *cinerea* in a number of hosts plants including pears (Meyer *et al*. 2000), strawberries (Mehli *et al*. 2005), grapes (Ricker *et al*. 1991; Marois *et al*. 1994; Obanor *et al*. 2004; Dewey *et al*. 2005; Dewey *et al*. 2008; Celik *et al*. 2009), boysenberry (Obanor *et al*. 2002) and cut flowers (Salinas and Schots 1994). Myer *et al*. (2000) conducted a trial comparing traditional media based isolation techniques with an ELISA based method for the detection of *B*. *cinerea* in pear stems during storage. The study found that the ELISA method was more sensitive and quicker than the traditional isolation techniques using incubation and media in detecting the latent infections in the fruit.

The ELISA tests that have been developed for the use in wine grapes have been targeted mainly for use at harvest at the weighbridge to give the estimated concentration of *B*. *cinerea* in the juice at crushing (Dewey *et al*. 2005). The aim of this is to provide a quick assessment as the grapes are transferred to the crusher so that appropriate additions can be made to minimise the must degradation that the fungus causes, or the fruit itself can be kept separate (rejected/ moved to separate tank). The kits developed by Dewey *et al*. (2005) use the *B*. *cinerea* antibody BC-12.CA4 and were designed to be a quick but semi-quantitative method to assess the level of BBR

present in the grapes. However, these kits are designed to be used during the later stages of ripening close to harvest and on wine, and not on fruit that is still going through the early berry development phase.

Where sensitive and more accurate detection is warranted, ELISA-based assays are being replaced with molecular-based methods (Boonham *et al*. 2008). This is because of a number of factors relating to the ELISA based method. As highlighted here, antibodies need to be created and the method is time-consuming, as well as requiring readily accessible animal hosts. In monitoring *B*. *cinerea* infections in stored table grapes, Celik *et al*. (2009) found that ELISA was not as reliable as qPCR for detection. The study found that the ELISA method worked well when the fungus was actively growing and symptoms were visible, as opposed to the qPCR, which was able to detect *B*. *cinerea* DNA on the day of inoculation of the table grapes or during early stages of colonisation. In the detection of *B*. *cinerea* in grapes, researchers are continually assessing methods to detect and quantify the fungus during the earlier developmental phase of the grape, rather than just at harvest when management practices cannot be implemented (Cadle-Davidson 2008; Scott *et al*. 2010; Evans 2011).

1.10.2. Polymerase Chain Reaction (PCR) & quantitative PCR

Polymerase Chain Reaction (PCR) is a technique that amplifies a specific segment of DNA using enzymes (DNA polymerase), deoxyribonucleotides and primers (short oligonucleotides) in specialised equipment programmed to cycle through set steps of denaturation, renaturation and DNA synthesis (Glick and Pasternak 1998). Real-time quantitative PCR (qPCR) is a modification of the PCR technique that allows not only amplification of the targeted DNA sequence, but also estimates their concentration (Wilhelm and Pingould 2003; Coolong *et al*. 2008). Therefore, real-time qPCR both detects and quantifies the amount of pathogen present in a sample. The technique involves constant monitoring of the reaction that is taking place within the reaction tube by detecting and quantifying the amount of target DNA in a sample using fluorescence. A value is only recorded when there is a statistical increase in the fluorescence signal from product formation during each cycle; this value is referred to as the Cycle Threshold or Ct value (Ward *et al*. 2004; Smith and Osborn 2008).

There are two main groups of qPCR assays, which are either amplicon sequence non-specific methods (SYBR Green I, ethidium bromide) or amplicon sequence specific methods (TaqMan®, Molecular beacons and Scorpion-PCR) (Wilhelm and Pingould 2003; Schena *et al*. 2004).

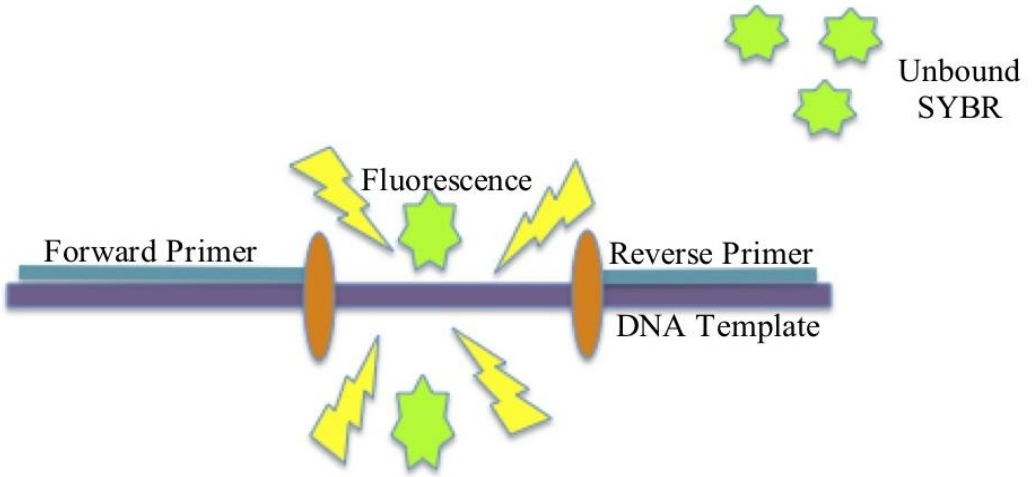

Figure 1.4: Pictorial representation of the SYBR qPCR reaction based on that of Wilhelm and Pingould (2003) and Smith and Osborn (2008). The SYBR dye binds/ intercalates with the double stranded DNA template when the primers bind to the DNA target.

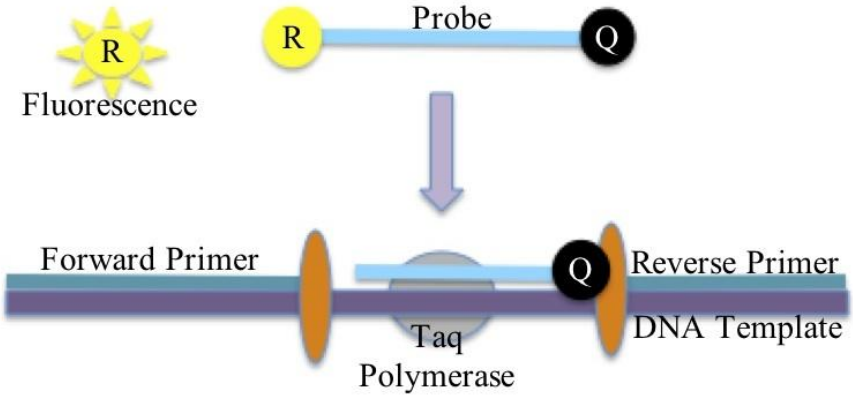

Figure 1.5: Pictorial representation of the Taqman® (hydrolysis) probe qPCR reaction based on that of Wilhelm and Pingould (2003) and Smith and Osborn (2008). The reporter dye/ fluorophore is represented by the yellow circle (R) and the quencher at the 3' is the black circle (Q). The reporter is released after the probe binds to the DNA template, resulting in fluorescence.

The most common example of the nonspecific qPCR assay is the SYBR Green based technique (Schena *et al*. 2004; Wilhelm & Pingould 2003). SYBR Green I is a fluorescence dye based detection method (Schena *et al*. 2004; Wilhelm & Pingould 2003). It works on the basis that when the dye intercalates or binds with double stranded DNA it fluoresces, but when it is free it exhibits very little or no fluorescence (refer to Figure 1.4) (Schena *et al*. 2004; Wilhelm & Pingould 2003). Therefore, as the dye requires double stranded DNA in order to fluoresce, the fluorescence intensity has a direct relationship with the amount of target DNA allowing quantification. The SYBR Green I reaction mix only requires the addition of target-specific primers and the SYBR Green dye, which reduces costs associated with the method, resulting in the method being more commonly used. As this method is a non-specific approach due to its ability to bind to any amplicon sequence, SYBR Green 1 requires the use of primers that are very accurate in targeting the specific sequence otherwise nonspecific amplicon sequences will fluoresce leading to a result that is unreliable (Gachon *et al*. 2004; Schena *et al*. 2004).

Amplicon sequence-specific methods work on the basis of oligonucleotide probes which are labelled with a donor fluorophore (molecule that absorbs light energy and becomes excited) and a quencher (acceptor dye) (Livak *et al*. 1995; Schena *et al*. 2004). These sequence-specific based methods are more accurate, as for fluorescence to occur, specific hybridization between the probe and the targeted DNA sequence is required, unlike SYBR Green I where the dye binds to the entire DNA (Gachon *et al*. 2004; Schena *et al*. 2004). The TaqMan® assay requires a sequence-specific probe that contains a fluorophore (reporter dye) at one end (5' end) and a quencher at the other end (3' end), which separate upon amplification, resulting in fluorescence only when the specific target amplicon is amplified (refer to Figure 1.5) (Wilhelm and Pingould 2003; Schena *et al*. 2004, Smith and Osborn 2008). TaqMan® probes are also commonly referred to as hydrolysis probes due to the nature of the reaction that takes place (Wilhelm and Pingould 2003).

Real-time qPCR was developed for use in the medical field to rapidly identify and quantify specific types of cancer cells, viral loads or genotypes in a given sample of tissue or body fluid (McCartney *et al*. 2003; Wilhelm and Pingould 2003; Boonham *et al*. 2008). Due to its reliability and robustness, it is also used to determine the cause

of food contamination (Schmittgen 2001; Wilhelm and Pingould 2003). Since development, it has been rapidly adopted in plant pathology, particularly for quantification of plant viruses and bacteria (e.g. *Xylella fastidiosa* that causes citrus variegated chlorosis or CVC) (Oliveira *et al*. 2002; Schena *et al*. 2004). It is increasingly being used in the study and identification of fungal plant pathogens, for example to quantify the causal agent of root disease of ginseng, *Cylindrocarpon destructans* f. sp. *panacis*, in soil, and the pathogen *Fusarium solani*, in soybean root samples (Gao *et al*. 2004; Kernaghan *et al*. 2007).

As previously stated, *B*. *cinerea* enters a latent phase after the initial infection during grape flowering or berry development, it is at this stage that methods for detection are limited. Unlike the ELISA tests, qPCR can be applied at all plant stages, which might make it ideal for monitoring latent infections. For the wine industry, developing a robust method in which latent infections of *B*. *cinerea* can be detected could enable industry to make more strategic decisions concerning management and fungicide use. For research, the qPCR method can become a tool in which researchers are able to accurately detect and monitor infections during all stages of fruit growth enabling a better understanding of the different phases of growth of the fungus.

Currently qPCR-based detection of *B*. *cinerea* in fruit is still in the early stages of development, especially in grapes, but there is great potential for these techniques to be adopted and modified. Several different genes and non-genic regions of the *B*. *cinerea* genome have been used in designing qPCR primers and probes (Rigotti *et al*. 2002; Suarez *et al*. 2005; Celik *et al*. 2009). These regions and genes include the β-tubulin gene, the IGS region (the intergenic region shown to be specific to *B*. *cinerea* genome) and RNA (Rigotti *et al*. 2002; Suarez *et al*. 2005; Celik *et al*. 2009). The method has been applied in detecting the fungus in grape berries in several published studies (Cadle-Davidson 2008; Celik *et al*. 2009; Diguta *et al*. 2010; Sanzani *et al*. 2012). The Celik *et al*. (2009) study investigated the use of qPCR and ELISA to track colonisation/ development of *B*. *cinerea* in table grapes during storage. As stated previously, the qPCR method was able to detect and track the colonisation of the fruit by *B*. *cinerea* earlier and more consistently than the ELISA method. The ELISA method was only able to detect the fungus during the later stages of infection when

symptoms started to appear. The qPCR assay used was SYBR based where there is an increased risk of fluorescence occurring due to non-specific nature of the assay. Like Celik *et al*. (2009), Diguta *et al*. (2010) used a SYBR based assay to quantify *B*. *cinerea* infection in wine grapes collected at harvest ripeness that were subjected to different fungicide treatments during the season. The assay was able to detect *B*. *cinerea* for all but one of the treatments, demonstrating that the ability of a qPCR method to detect *B*. *cinerea* in samples is not hampered by fungicide application. It was also able to identify differences in the level of efficacy among fungicide treatments. However, these two assays did not look into using the qPCR technique to study and track infections from initial colonisation in developing fruit.

The potential for qPCR to be used as a tool to track latent infections have been explored by Cadle-Davidson (2008) in preliminary studies. The method used by Cadle-Davidson was a TaqMan® based qPCR, in which primers were designed based on the *B*. *cinerea* sequence published by Rigotti *et al*. (2002). The study also investigated the use of previously published primers by Rigotti *et al*. (2002) in a SYBR based assay and the use of moist incubation. Cadle-Davidson (2008) conducted the trial over two seasons using 32 *Vitis* accessions, collecting samples from randomly selected vines from a research station vineyard. The qPCR results, in comparison with the moist incubation, were found to be more reliable in the detection of *B*. *cinerea* in the grape berry samples over the two growing seasons. The first season was more conducive to *B*. *cinerea,* resulting in higher disease levels relative to the second season, which was much drier. The study also highlighted the risk in using SYBR based assays relative to the more sensitive Taqman® probe based assays. The SYBR method was unable to reliably detect the lower levels of *B*. *cinerea* infections, due to positive results caused by non-specific fluorescence that can occur even in comparison with the negative grape DNA control due to the design of the reaction. Further testing and development of the qPCR technique for the quantification of BBR may in the future lead to a better test that can reliably quantify the disease in samples and rely less on the visual assessments where often it may be too late to limit a disease epidemic. Sanzani *et al*. (2012) also investigated the use of qPCR to detect latent infections in the table-grape red globe at harvest and prior to storage, using the same IGS region as Suarez *et al*. (2005), however like Cadle-Davidson they also used an internal control using primers designed to detect *V*. *vinifera* DNA from a study by

Savazzini and Martinelli 2006). The study found that they could detect latent infections in harvest ripe fruit from the region between the pedicel and the berry and the stamens still attached to the rachis when the fruit was picked. However unlike Cadle-Davidson (2008), the standards developed in this study used water as a dilutant but did spike each standard with the same amount of *V*. *vinifera* DNA, rather than using a *V*. *vinifera* DNA stock as a dilutant. Both of these studies also noted that the qPCR method could be more reliable than that of the moist incubation method, which is more time consuming and has a longer turnaround time than that of qPCR.

1.10.3. Loop-mediated isothermal amplification

Loop-mediated isothermal amplification or LAMP is molecular tool, which does not require thermal-cycling method like PCR (Tomlinson *et al*. 2010). However the reaction additional to the DNA sample requires the addition of four to six primers and DNA polymerase that has stand displacing activity in order to generate amplification products that contain single-stranded loops, allowing primers to bind, but there is no cycling (Notomi *et al*. 2000; Nagamine *et al*. 2001; Tomlinson *et al*. 2010). The primers are designed similar to that of ones used for PCR. The first set of forward and reverse primers (internal primers) bind to the target, then the second 'external' set of primers interact with the strand that has bound to the target, displacing the DNA strands, that contain the stem-loop structures. The third set of primers (loop primers) is then used to accelerate the amplification by binding the loops of which are in incorrect orientation to bind with the internal primers (Nagamine *et al*. 2002). To quantify the amount of target, there are a number of options, which include gel electrophoresis, observation of precipitated magnesium pyrophosphate (by product of amplification (Mori *et al*. 2001); spectroscopy using colour-changing reagents (Goto *et al*. 2009); or via the use of LFDs (lateral flow devices) to detect fluorescence, when dyes are incorporated into the products during or after the amplification (Tomlinson *et al*. 2010b) or measuring the amount of fluorescence or turbidity (Mori *et al*. 2004; Maeda *et al*. 2005). Tomlinson *et al*. (2010a)

1.10.4. The application of spectroscopy

Spectroscopy is used to measure absorbance of light across a range of wavelengths, by a substance that can be in solid, liquid or gas form. There are four possible wavelength ranges used to measure the electromagnetic spectrum of a sample which include ultraviolet (UV) (200 - 400 nm), visible (VIS) (400 - 700 nm), near infrared (NIR) (750 - 2,500 nm) and mid infrared (MIR) (2,500 - 25, 000 nm) (Cozzolino *et al*. 2007b; Gishen *et al*. 2010). In the wine industry, research has been conducted to produce methods for rapid analysis of wine, juice and grapes for quality control (Gishen *et al*. 2005; Cozzolino *et al*. 2007a). The advantage of this type of technique is that it requires very little sample preparation as it either requires small sample volumes or can be non-destructive (i.e. bottle wine analysis) (Gishen *et al*. 2005; Cozzolino *et al*. 2007b; Gishen *et al*. 2010). To measure fungal colonisation in grapes, spectral methods are still in developmental stages, and research to date has been conducted in powdery mildew and BBR infections of grapes (Cozzolino *et al*. 2003; Dambergs *et al*. 2007; Versari *et al*. 2008; Gishen *et al*. 2010; Scott *et al*. 2010).

Prior to the adoption of the method in the wine industry, spectroscopy methods have been widely used in agriculture. It has been used to assess grain quality via measuring protein and moisture (Batten 1998), contamination by powdery mildew and rust (Asher *et al*. 1982), fungal toxins in wheat (Dowell *et al*. 1999) and fungal contamination and toxins in corn kernels (Peason *et al*. 2001; Kos *et al*. 2004). In viticulture, the method has been examined for use in determining vine water status where NIR was used to determine stem and leaf water potential (Tyerman *et al*. 2007). To assess grape and wine quality, methods have been developed to simultaneously measure total soluble solids and pH, with the main commercial application being determination of anthocyanin (pigment) and tannin content in red grapes and wine (Kennedy 2002; Cozzolino *et al*. 2004; Gishen *et al*. 2005; Cozzolino *et al*. 2010a; Cozzolino *et al*. 2010b; Gishen *et al*. 2010). Recently there have been investigations into non-destructive methods of analysing bottled wine during storage (Ugliano *et al*. 2010). Versari *et al*. (2008) used the MIR wavelength to measure gluconic acid and glycerol present in grape samples taken at harvest. These two chemical compounds result from *B. cinerea* infection. The study used Fourier-

Transformed MIR (FT-MIR), which measures all wavelengths simultaneously within the MIR range, after which complex data analysis is completed. The study found that the amount of these two chemical compounds correlated with the visual assessments. Currently the use of these methods for measuring BBR level is limited to research as there is yet to be standard protocols published that can be readily adopted and used by industry (Scott *et al*. 2010).

1.11. Rationale for project:

This project was comprised of five objectives to build on the current knowledge base of BBR. The first objective was to build on the previous work by Cadle-Davidson (2008), with the further development and application of a qPCR as a research tool to study the temporal progression of *B. cinerea* from early berry development up until harvest. To date there has been limited studies where accurate quantification and understanding of the temporal progression of the disease have been completed. The second objective was to investigate other methods of measuring BBR load in grapes in comparison with the traditional visual scoring method. The third objective was to investigate the role of spray timing in the control of BBR. The fourth objective in combination of the third objective was to investigate the key infection pathways for Tasmania, via investigating latent infections and the role of floral remnants. The fifth objective was to use the technique of precision viticulture in regards to the application of the whole of block experimental method, to investigate the spatial-temporal dynamics of BBR, and the vineyard factors that may contribute to the expression of disease and the effective use of fungicides.

CHAPTER TWO
Method development for the quantification of *Botrytis cinerea* in wine grapes

2.1 Introduction

Real-time quantitative PCR (qPCR) unlike conventional PCR can be used to accurately quantify the amount of target DNA in a sample during the PCR with data collated in real-time, eliminating the time consuming step of gel electrophoresis (Wilhelm and Pingould 2003). This technique has been adopted for use in the identification and quantification of a number of fungal and viral plant pathogens, including *Phytophthora ramorum* (sudden oak death), *Phytophthora cryptogea* (root rot in flowers) and various citrus viruses (Hayden *et al*. 2006; Minerdi *et al*. 2008; Loconsole *et al*. 2010). The technique has been applied in the study of other economically important grape diseases, which include powdery mildew (*Erysiphe necator*) (Stummer *et al*. 2006), downy mildew (*Plasmopara viticola*) (Valsesia *et al*. 2005) and the mycotoxin producing fungus *Aspergillus carbonarius* (Selma *et al*. 2008). They have the ability to eliminate the for the use of time consuming methods of either assessing the plant for symptoms or incubation of plant samples for isolation of the causal agents on media that supports pathogen growth (McCartney *et al*. 2003; Ward *et al*. 2004). The methods can also be applied in asymptomatic tissue and detect latent infections (McCartney *et al*. 2003; Gachon *et al*. 2004; Ward *et al*. 2004).

For the study and detection of *B*. *cinerea* there has been a move to develop qPCR assays for a number of crops including onions (Coolong *et al*. 2008), strawberries (Mehli *et al*. 2005), *Pelargonium* spp. and other flower species (Suarez *et al*. 2005). Recently there have been several qPCR assays published for the quantification of *B*. *cinerea* in grape samples (Cadle-Davidson 2008; Celik *et al*. 2009; Diguta *et al*. 2010, Sanzani *et al*. 2012). Both Cadle-Davidson (2008) and Celik *et al*. (2009) used the qPCR assays that they developed to study the colonisation of *B*. *cinerea* in grape berries. However, these assays did not take into consideration that a negative result

could be due to the presence of PCR inhibitors and that the DNA samples would be a mixture of both host (*V. vinifera*) and pathogen (*B. cinerea*) DNA, as the assays were simplex in design. The plant's cells contain molecules that include polysaccharides, polyphenols and proteins, all of which can end up becoming contaminates in the DNA sample after the extraction process causing PCR inhibition (Varma *et al*. 2007). Simplex qPCR reactions consist of only one set of primers for the target organism, whereas duplex have two sets, where the second primer/probe set is used as a control to detect a second DNA target. The study by Sanzani *et al*. (2012) developed and tested a duplex assay to detect latent infection in harvest ripe table grapes, where they designed a primer and probe set to detect latent *B. cinerea* but used a published qPCR primer and probe set to detect *V. vinifera* DNA as a control (Savazzini and Martinelli 2006). The study found that it detected *B. cinerea* DNA in 80% of the samples taken from the area around the peduncle (stem that is connected to the berry). However, the assay did not test whole berries, unlike the Cadle-Davidson study (2008). The advantage of the method is that when there is a negative result for the target organism, amplification of the second target can validate the result ensuring that the result was not due to PCR inhibition.

Like any new method, during the developmental and testing phase of a new qPCR assay there are a number of decisions and steps that have to be completed prior to its application (Bustin *et al*. 2009). For qPCR assays currently there is either SYBR or probe-based chemistries available (Gachon *et al*. 2004; Schena *et al*. 2004). The SYBR-based assays are used readily due to being cheaper and only require the design of PCR primers. However, using probe-based chemistries can reduce the risk of a signal being produced from other artefacts such as primer dimers and non-specific amplification, which is a potential risk with SYBR-based assays (Ward *et al*. 2004). In a probe-based reaction, for the PCR machine to detect the target DNA, the probe is required to bind to the target DNA sequence after and between the forward and reverse primers (Wilhelm and Pingould 2003; Schena *et al*. 2004; Ward *et al*. 2004). There are several types of probes available and, as the technology and methods develop, further types will become available as biotechnological companies and researchers look for continued improvement in the overall techniques. One common style of probe that is commonly used is categorised as a hydrolysis probe, an example of which is the Taqman® probe (Wilhelm and Pingould 2003). The Taqman® probe is

a labelled oligonucleotide which contains a reporter fluorophore, that is a fluorescence dye such as FAM (6-carboxy fluorescein) or TAMRA (6-carboxy-tetra-methyl-rhodamine) at the 5' end of the sequence and a quencher (not fluorescent) either at the 3' end or internally in the sequence or vice versa for each end (Nazarenko *et al*. 2002; Wilhelm and Pingould 2003; Dorak 2010). When the probe binds to the sequence, the quencher is released from the fluorophore resulting in the fluorescence (Wilhelm and Pingould 2003).

An advantage associated with probe-based qPCR is that several reactions can take place simultaneously within the one reaction tube due to the machine having several different spectral channels (light emitters). The advantage of this feature is that a second reaction can be designed to target DNA that that will either be co-extracted or used to spike the test DNA sample. This second reaction indicates that when there is a negative result the cause is not PCR inhibition but rather that the target DNA is absent and the result is truly negative. When PCR inhibition occurs it is often the result of molecules being co-extracted during the DNA extraction process. However, in designing a second set of primers and probes to work with the primers and probe designed to detect the target DNA, optimal annealing temperatures for both must be as close as possible for the reactions to work within the one sample. Most qPCR reactions fall into three groups with regards to assessing a negative result: - 1) internal control that is detected simultaneously with the target DNA; 2) detection of a control in a secondary PCR reaction; or 3) no control at all.

Quantification of the amount of target DNA within a sample involves comparing the result with a set of standards (i.e. a dilution series) containing known concentrations of the target DNA. Generation of a standard curve for a qPCR assay involves serial dilution of a standard stock solution of purified target DNA with the aim of producing a fitted line with a high R^2 value in which Ct values are plotted against the known concentrations. The amount of the target DNA in the unknown test sample can then be estimated with known accuracy. Components of the qPCR reaction, including final cycling conditions, primer and probe concentrations, DNA amount and reaction volume can be optimised to achieve a high R^2 value and a high reaction efficiency prior to the application of the assay to test samples. The slope of the linear equation derived from the samples is used to calculate the reaction efficiency. It is a measure

used to describe the exponential amplification that occurs within a qPCR reaction. The optimal range of the calculated reaction efficiency for a qPCR run is between 90 – 110% (Bustin 2004; Dorak 2010). The efficiency is calculated on the assumption that after the completion of each cycle during the cycling process, the amount of amplicon in the PCR tube doubles (Bustin 2004; Dorak 2010). The efficiency is then calculated using the linear equation derived from the standard dilution series used in the qPCR run, where the calculated slope value should be between – 3.1 and – 3.6 (Bustin 2004; Dorak 2010). Another important aspect of designing and using a qPCR assay is the sensitivity. This is often referred to as the limit of detection and is expressed as the lowest concentration at which an assay can detect the target within a sample (Bustin *et al*. 2009).

The application of qPCR has the potential to provide a better understanding of pathogen biology by accurately quantifying the target organism within a plant in which symptoms are not always visible. The first objective of this study was to evaluate the published Taqman® qPCR assay developed by Cadle-Davidson (2008) for the detection of *B*. *cinerea* in grape berries, which was based on the same intergenic region used by Rigotti *et al*. (2002). A study trip to the USA enabled the examination of the Cadle-Davidson (2008) assay in comparison with those of Suarez *et al*. (2005) and Rigotti *et al*. (2002) prior to the commencement of experimental work in Australia, which found that this assay was better suited for the detection *B*. *cinerea* in grape berry samples (data not shown). The second objective was to develop and evaluate a duplex Taqman®-based assay for concurrent quantification of *B*. *cinerea* and *V*. *vinifera* DNA for eventual application of the technique to samples from the vineyard. The decision to develop a new duplex Taqman®, rather than evaluate previously published assays was based on the need to minimise the risk of non-specific binding and background fluorescence that result in false positives, which is a greater risk when using SYBR chemistry (Gachon *et al*. 2004; Schena *et al*. 2004; Celik *et al*. 2009; Diguta *et al*. 2010).

2.2. Methods

2.2.1. Collection and preparation of plant and fungal material

2.2.1.1. Origin and processing of grape leaves

Healthy grape (*Vitis vinifera*) leaves were sourced from micropropagated 'Pinot Meunier L1 Dwarf' plants (CSIRO Material Transfer Agreement 2008020619, Adelaide, South Australia), from potted Riesling or Chardonnay vines sourced originally from the Clare Valley, or from Chardonnay vines grown in a commercial vineyard in Tasmania. The micropagate vine material was maintained on 50 mL of MS media in 250 mL polycarbonate culture tubes with polypropylene lids (Techno-Plas). The recipe of the media is that of Evans *et al*. (1996) and Murashige & Skoog (1996). The media consisted of ½ strength MS salts and nutrients (4.4 g/L) (Sigma Aldrich) (Murashige & Skoog, 1996); sucrose (1.5%) (Sigma Aldrich), and agar (Oxoid Australia Pty Ltd). The pH was adjusted to 5.7 using NaOH (MP Biochemicals) (refer to Appendix A for actual amounts). The microprogated vines were kept in an incubator set at 25 °C with a 16-hour Light period. Plant material, sourced from either potted vines or the vineyard, consisted of small, healthy, unexpanded grape leaves, due to the expected higher quality and yield of DNA likely to be obtained relative to older, larger, expanded leaves (Varma *et al*. 2007). Compared to older leaves, younger leaves contain lower concentrations of polyphenols, tannins and polysaccharides (Varma *et al*. 2007) that might inhibit the PCR. Once the leaves were harvested, they were stored in a -80°C freezer until DNA extraction. Plant material was ground in liquid nitrogen using a mortar and pestle to obtain approximately a 500 µL volume of tissue.

2.2.1.2. Origin and processing of fungal tissue

A bulk isolate of *B. cinerea* was obtained by moist incubation of dead rachii sourced from Sauvignon Blanc vines in a vineyard in the Coal River Valley of Tasmania in winter (June 2007). After several days of incubation, pieces of plant material with signs of *B. cinerea* were transferred aseptically to half-strength lactic potato dextrose Agar (Oxoid Australia Pty, Ltd, Adelaide) (LPDA) in 9 cm diameter Petri plates

(refer to Appendix A for media preparation). The method used to prepare media was that of Shurtleff & Averre (1997). Fungal mycelia with characteristics of *B. cinerea* were transferred aseptically several times to fresh plates containing LPDA. The purified isolate (KD0707) was transferred to malt extract agar (MEA) (Oxoid Australia Pty Ltd, Adelaide) and then onto full strength potato dextrose agar (PDA) (Oxoid Australia Pty Ltd, Adelaide) followed by long-term storage on PDA slopes at 4 °C. The isolate was multiplied on MEA at room temperature with a 12-h photoperiod from cool white fluorescent lights, which also included a Gro-Lux tube that produced light in the near UV range. Fungal material containing spores and mycelia was scraped off the agar of two full plates using a razor blade and transferred into several 2 mL Eppendorf tube which was then snap frozen in liquid nitrogen prior to DNA extraction.

2.2.1.3. Field sample collection and processing

During the 2007-08 growing season, a replicated small plot trial (refer to Chapter Four) was used to obtain berry samples to test the application of the qPCR assay. The randomly tagged bunches used for sampling were selected prior to flowering during the initial trial setup to distinguish bunches from those which were used for visual assessment as part of the trial. A single berry per bunch over 12 bunches were harvested over time. The number of berries/bunches was selected based on limitations of bunches available, given that bunches were repeatedly sampled during the season. Samples were taken from the two end vines of each treatment panel (five vines); each vine had a total of twelve bunches tagged. During harvesting and transportation, the berry samples were kept in Styrofoam boxes containing ice packs until transfer to a -20 °C freezer and storage for up to 12 months. Prior to DNA extraction, each berry sample was transferred to a linen/calico bag and submerged in liquid nitrogen prior to pounding the bags with a rubber mallet to break the berries into small pieces. Bags were folded over to prevent berries falling out during the initial breaking. The small berry pieces were then transferred to a mortar and pestle for grinding in liquid nitrogen to produce a fine powder. Two 500 µL tubes (Astral Scientific, Adelaide) were filled to the top with the ground berry samples and stored

in liquid nitrogen until transferred to -85 °C freezers, where they were kept for up to 7 days until DNA extraction.

2.2.2. DNA extraction protocol adapted for Australian laboratory equipment

The DNA extraction method used was that of Cadle-Davidson (2008) which is based on the method originally developed by Lin & Walker (1997). The method was modified by Cadle-Davidson (2008) for the extraction of grape berry tissue and to suit the use of 96 well plates/racks. For the extraction of DNA from the *V. vinifera* and *B. cinerea* standards, 2 mL microfuge tubes were used instead of plates. Three stock buffers were made up and will be referred to as Buffer A, Buffer B and Buffer C (refer to Appendix A for solutions). To assist in the tissue breakdown, sterile (autoclaved and soaked three times (1 min intervals) in 70% ethanol) 3 mm stainless steel beads (CBG Precision Products, Melbourne, Australia) were placed in the tubes prior to the addition of *B. cinerea* mycelia, leaf material or a field grape sample. A refrigerated Eppendorf centrifuge (model SW417R, Eppendorf, Hamburg, Germany) was used for each of the centrifugation steps during the DNA extraction of the DNA standards (*B. cinerea* and grape DNA) and settings were 21, 000 RCF. For the DNA extraction of the field samples a Sorvall Super T21 SL5OR centrifuge (Thermo Fisher Scientific, Waltham MA USA) was used for all centrifugation steps set at 3, 800 RCF.

The 500 µL tubes (Astral Scientific) containing the ground grape tissue from the vineyard were taken out of the -80 °C freezer and kept in liquid nitrogen to prevent thawing. The base of the tubes were clipped off and the material pushed through to a 1.2 mL costar cluster strip tube within a 96 well rack (Corning Inc., Lowell MA, USA) using a pipette tip, aided by addition of 750 µL of Buffer A modified with 3 % w/v polyvinylpyrrolidine (PVP40) (Sigma Aldrich) and 0.1 % w/v beta-mercaptoethanol (Sigma Aldrich). After the transfer of 96 samples to the strip tubes in the rack, all samples were homogenised using a Retsch Plate Shaker (MM200) (Retsch, Hann, Germany) set at 30 vibrations/s for 30 s. Samples were frozen with liquid nitrogen and placed back in the - 80°C freezer prior to commencing the next step in the extraction process. For the extraction of the tissues for the DNA standards,

750 µL of the modified Buffer A was directly added to the 2 mL tubes and the mixture re-frozen using liquid nitrogen after the initial homogenisation, as described previously.

The frozen racked samples with the modified buffer A were taken out of the - 80°C freezer and left to thaw at room temperature for 10 min, followed by shaking using the Retsch shaker set at the 30 vibrations/ s for 5 min. Samples were then left to settle briefly, followed by a second homogenisation step to ensure sample had defrosted and mixed thoroughly. This initial thawing step was not used for the *V. vinifera* and *B. cinerea* standards as the tissues were transferred straight into the Eppendorf tube and the modified Buffer A added. The samples were then centrifuged for 20 at 4ºC (RCF settings as mentioned above). After centrifugation, the supernatant, containing lipids and polysaccharides, was discarded and 200 µL each of Buffer A containing 0.1 % beta - mercaptoethanol, Buffer B and Buffer C were added and the samples homogenised using the shaker for 1 min. Samples were then incubated in a water bath at 75°C for 30 min, initially using the shaker function but then shaking by hand at 10 min intervals to allow the solutions to break down proteins and cell structures. Samples were then left to cool for 5-10 min prior to the addition of 200 µL of a solution containing 24:1 chloroform (MP Biochemicals) and isoamyl alcohol (Sigma Aldrich). Samples (either Costar plates or Eppendorf tubes) were shaken using the shaker for 15 s to ensure maximum homogenisation followed by settling for 5 min. This step was then repeated by hand shaking and settling, prior to samples being placed in their respective centrifuges (RCF settings mentioned above). Timing of the centrifuge step was 20 mins and the temperature set at 4ºC. The purpose of this step was to separate the proteins and other materials not removed in previous steps from the aqueous solution.

The aqueous phase was then collected and transferred with a pipette into clean 1.2 or 2 mL tubes. Another 200 µL of the chloroform: isoamyl solution was added to separate proteins and other compounds that were not removed previously. Samples were shaken followed by 5 min of settling, with this step being repeated as per the first chloroform: isoamyl addition, except that both sets of shaking were performed by hand, after which samples were placed in their respective centrifuges (settings as mentioned above). A volume (400 µL) of the supernatant was collected and 800 µL

of 95 % ethanol was added. Samples were shaken by hand and placed in the freezer (-20 ºC) overnight to precipitate the DNA. Samples were then spun at room temperature in the respective centrifuges until the centrifuge had reached 3800 RCF for the field samples and 21, 000 RCF for the standards or until the DNA pellet was visible (approximately 5 mins). The supernatant was then poured off and the DNA pellets were left to dry for 10 min prior to washing twice with 70 % ethanol (MP Biochemicals) (room temperature). After the washing steps, the racks of field DNA pellets were then left in an oven set at 37°C until dry, ensuring total removal of any excess ethanol left behind after the decanting. The DNA pellet in 2 mL tubes was dried using a DNA mini dehydrator (Imbros, Pty Ltd, Tasmania). Each DNA pellet was then dissolved in 35 µL of sterile Milli Q water.

After initial testing of the duplex qPCR assay, the DNA extraction method for all DNA samples (*B. cinerea*, *V. vinifera* leaf and field grape samples) had to be modified to reduce the presence of PCR inhibitors that may have co-extracted in the initial process. After the DNA pellets had been dissolved in the sterile Milli Q water, the samples were purified further using an Ultra Clean® 15 DNA Purification Kit (MoBio Pty Ltd, Carlsbad, CA, USA) as per the manufacturer's protocol. The centrifuge speeds were the same as before, with centrifugation at room temperature. After further testing it was found that a second cleaning step had to be applied to the *V. vinifera* and grape field DNA samples; this step involved using SureClean (Bioline Pty Ltd, UK). Modifications were made to the manufacturer's protocol to ensure maximum DNA yield, as follows. The initial incubation time after the addition of the buffer was increased to 20 min, the centrifugation time was also increased to 20 min. An additional cleaning step using 70 % ethanol was added to ensure the maximum amount of PCR inhibitors were removed, as recommended by the manufacturer.

2.2.3. DNA quantification

All DNA samples were quantified using the Quant-iT PicoGreen Assay (Invitrogen, Australia) and a real-time PCR machine (RotorGene 6000) (formerly Corbett Life Sciences, now Qiagen Pty Ltd), following the manufacturer's recommendations. The quantification was performed using 3 µL of DNA sample, which was diluted with 47

µL of 1X TE buffer (Invitrogen, Australia). The diluted PicoGreen dye, which was made up as per the manufacturer's specifications, was then added to the diluted DNA samples. Quantification was performed in duplicate using 25 µL sub-samples of the diluted DNA containing the PicoGreen dye and the RotorGene real-time qPCR machine was programmed to run the specific settings for DNA quantification which included a 2 min hold step at 50 °C followed by 10 cycles of 5 s at 50 °C. Samples were quantified by comparison with a set of seven dilutions made up using the supplied calf thymus DNA stock and TE buffer (Quant-iT kit). All samples with a concentration of DNA above 5 ng/µL, were then diluted to this value using sterile Milli Q water.

2.2.4. Adoption of Taqman® *qPCR assay*

2.2.4.1. Adoption of technique

The Taqman®-based qPCR assay designed by Cadle-Davidson (2008) was practised in the USDA-ARS Grapes Genetics laboratory and adapted to the University of Tasmania laboratory conditions prior to adoption in Australia. The sequences used were as follows: forward primer (BcTaq424f) 5' GCT TCC CCC GTA TCG AAG A'3, reverse primer (BcTaq491r) 5' CGA ACG GCC AGG TCA TCT'3, and the Taqman® Probe (BcFamP) 5' -6-Fam CCC TAG ATT TA TTT TAC CCT TCG CGT GG BHQ-1'3 (Cadle-Davidson, 2008).

2.2.4.2. Optimisation of assay to suit equipment

A gradient PCR was set-up to determine the optimal annealing temperature for the Taqman® assay described by Cadle-Davidson (2008) using a total reaction volume of 20 µL. Two reaction mixes were made to test the primers with and without the associated probe. The first mix contained 10 µL 2× Hot StarTaq PCR mix (Qiagen, Valencia CA, USA) and 1 µM of each primer (Integrated DNA Technologies, Coralville IA, USA) per reaction. The second mix contained the same reaction mix plus 1 µM of each primer and 1 µM of the probe (Integrated DNA Technologies, Coralville IA, USA). Each reaction included 2 µL of a sample container either 10 ng of *B. cinerea* DNA, 10 ng of Pinot Meunier DNA, 10 ng of a second Pinot Meunier

DNA stock or RNase- free water (Qiagen). The volume was made up to 20 µL using the RNase-free water. A second Pinot Meunier DNA sample was tested to ensure DNA stock was clean and to ensure a negative result.

Reactions were set up using a 96 well PCR plate with 200 µL wells sealed using a sterile silicon mat prior to PCR using a Master Cycler Gradient PCR Machine (Eppendorf, Hamburg, Germany). Cycling conditions were based on Qiagen Pty Ltd recommendations and included the following: - an initial step of 15 min at 95°C followed by 35 cycles of 95°C for 30 s and the gradient annealing temperatures of 55°C, 55.3°C, 56.5°C, 58.3°C, 60.6°C, 63.2°C, 65.9°C, 68.5°C, 71°C, 73.1°C, 74.6°C and 75.4°C for 30 s and an extension step of 72°C for 1 min, followed by a final extension step after the cycling for 10 min at 72°C.

The PCR product/s were then visualised by gel electrophoresis by running them on a 2 % agarose gel containing SYBR Safe staining dye (Invitrogen, Carlsbad CA, USA) in a 1X lithium borate buffer, for 20 min at 300 volts (refer to Appendix A for solution preparation). A Bioline Easyladder 1 molecular marker (Bioline, London UK) was run concurrently to check the approximate size of the PCR product. The optimal annealing temperature was determined based on the highest band intensity observed for the positive control (*B. cinerea* DNA) and the reaction mix containing both the forward and reverse primers and the probe.

2.2.4.3. Standard DNA dilution series used during method development

Two stock solutions of *B. cinerea* DNA and Pinot Meunier (grape) DNA were prepared to a concentration of 5 ng/µL. The two stock solutions were then used to create a series of solutions with varying concentration of *B. cinerea* DNA diluted in Pinot Meunier DNA. Cadle-Davidson (2008) explained that by using Pinot Meunier DNA as a diluting factor rather than water could reflect the likely components in the DNA samples of our study, which would come from the field and comprise of *V. vinifera* DNA and potentially *B. cinerea* DNA. For the dilution series, the first standard was prepared using a dilution factor of 1:1 of *B. cinerea* and Pinot Meunier DNA. The second standard was prepared using a 1:5 dilution factor of *B. cinerea* to

Pinot Meunier DNA. With the rest of the standards, one volume of the previous standard was diluted with 5 volumes of Pinot Meunier DNA stock dilution to obtain the concentrations shown in Table 2.1.

Table 2.1: Standard dilution series used during initial testing of the qPCR technique (Cadle-Davidson, 2008) showing the concentration of *B. cinerea* and Pinot Meunier DNA in each solution where the total DNA concentration was always 5 ng/µL. The total amount of *B. cinerea* DNA for each standard using a volume of 5 µL for each qPCR is also shown.

Standard name	Concentration of *B. cinerea* DNA (ng/µL)	Concentration of Pinot Meunier DNA (ng/µL)	Concentration of total DNA (ng/µL)	Amount of *B. cinerea* DNA (ng) in 5 µL
Botrytis	5	-	5	25
Standard 1	2.5	2.5	5	12.5
Standard 2	0.5	4.5	5	2.5
Standard 3	0.1	4.9	5	0.5
Standard 4	0.02	4.98	5	0.1
Standard 5	0.004	4.996	5	0.02
Standard 6	0.0008	4.9992	5	0.004
Standard 7	0.00016	4.99984	5	0.0008
Grape	-	5	5	0

2.2.4.3. Reaction mix comparison

In transferring the qPCR technique to the University of Tasmania laboratory, it was necessary to use reaction mixes that were designed to suit the Corbett's (now Qiagen) rotor style machine and not the traditional plate style that was used in the Cadle Davidson study (2008). The purpose of the experiment was to test the reliability of the assay using two different specific mixes for qPCR that were designed for use with the rotor style PCR machine.

The first mix tested was the SensiMix dT (Bioline, London, UK). The DNA samples used to test the reaction mix were *B. cinerea* (5 ng/ µL), standards 1, 4 and 7 from the dilution series (Table 2.1), Pinot Meunier DNA (5 ng/ µL) and *V. vinifera* (cv Riesling) DNA (5 ng/ µL). Each reaction had a total volume of 25 µL, which contained 2× master mix (SensiMix dT) (12.5 µL), 0.4 µM of each primer and 0.2 µM of the probe (Integrated DNA Technologies, Coralville, IA, USA), 25 ng (5 µL) of total DNA (*B. cinerea* DNA, grape DNA or mixtures of *B. cinerea* and grape DNA), with the final reaction volume achieved by addition of RNase-free water (Qiagen,

Valencia, CA, USA). The second reaction mix tested was RotorGene Probe qPCR sample kit (Qiagen, Valencia, CA, USA). The reaction volume was the same except that the Qiagen Reaction master mix was used. Controls comprised only *B. cinerea* DNA and water due to the limited amount of reaction mix sourced.
Assays for each reaction mix were repeated over three consecutive days using a different water source for each day. The water was sourced from the following: - on day 1 RNASE free water (Qiagen) was used; on day 2, autoclaved Milli Q water was used; on day 3, autoclaved water treated via filter sterilisation. All water was exposed to UV light using a UV crosslinker set at 1X 0.240 joules prior to opening.

The real-time PCR machine used to evaluate the reaction mixes was a RotorGene 6000 (Qiagen, formerly Corbett Life Sciences, Valencia, CA, USA). For both reactions, the number of cycles was the same (60 cycles) as in Cadle-Davidson's study (2008). The annealing/extension temperature of 58.3°C was selected based on the gradient PCR results (refer to section 2.2.4.2 for setup and 2.3.1.2 for the results). The cycling conditions for the SensiMix dT included an initial activation step of 95°C for 10 min, followed by 60 cycles of a denaturation step of 95°C for 15 s and an annealing/extension step of 58.3°C for 45 s. The cycling conditions for the Qiagen reaction mix were as follows: initial activation step of 95°C for 3 min, and then 60 cycles of a denaturation step of 95°C for 3 s followed by a combined annealing and extension step of 58.3°C for 10 s.

All reactions were analysed using the RotorGene 6000 software, and initial runs were checked via gel electrophoresis, as described previously for the gradient PCR analysis.

2.2.5. Re-design and adoption of duplex assay

After initial testing, the assay designed by Cadle-Davidson (2008) was found to be unreliable when used in conjunction with the RotorGene machine and reaction mixes that were initially tested (refer to Section 2.2.4.3 for reaction mix experiment). A number of factors may have contributed to the assay being unsuited to the available laboratory conditions. Further investigation of the sequences found that the assay had

the potential to produce hairpins, primer dimers and cross dimers, which can result from parts of the primers binding onto themselves and the probe partially binding to the primers; hence, the decision was made to design new primers and a probe using the same intergenic region and type. Analysis via computer software (Beacon designer, Premier Biosoft International) found that the original assay had an increased risk of both the forward and reverse primers forming a cross dimer with each other and with the probe, resulting in the increased fluorescence. It was also discovered that the probe had an increased risk of forming dimers within itself.

A new duplex Taqman® assay was designed incorporating a control to ensure that if a negative result was obtained in a field sample it was not due to PCR inhibition from poor DNA extraction, but rather because there was no *B*. *cinerea* DNA present. As *V*. *vinifera* DNA would be co-extracted from the field samples, the internal control was designed to detect the DNA in the samples when the qPCR technique would be applied. In consultation with Dr Fabrice Magnino from Integrated Sciences (Sydney), the primers were designed for the detection of *V*. *vinifera* DNA. A new set of primers were designed for the detection of *B*. *cinerea* based on the intergenic spacer (ITS) region used by Cadle- Davidson (2008) and initially sequenced by Rigotti *et al*. (2002) (NCBI database, accession number AJ539088). The new primers (labelled KJD from here on) resulted in amplifying a larger product of 150 bp compared to those from the initial assay (labelled LCD from here on) designed by Cadle-Davidson (2008) for which the product size was 67 bp (refer to Figure 2.1).

The primer sequences selected for the detection of *B*. *cinerea* DNA were as follows: forward primer (KJD BcF) 5'GGA CTT GGA CAT GGA TAC'3; reverse primer (KJD BcR) 5'ACA ATC AAA GAC CAG AGG'3 and the Taqman® probe (KJD BcP) 5'6-FAM CAC TCG CAC CTA ATT CGT CAA CG BHQ-1'3 (Eurogentec, Integrated Sciences Pty Ltd, Sydney). The primer sequences for the detection of *V*. *vinifera* DNA were designed based on the *V*. *vinifera* chromosome 10 (Jaillon *et al*. 2007) (reference sequence on NCBI NC_012016.2). Refer to Figure 2.2 for partial sequence of the gene and primers and probe position for *V*. *vinifera*. The primer sequences were as follows: forward (KJD GF) 5' GGC TGT TAA GGT ATA TGC T'3; reverse (KJD GR)-R 5'AAT TAC TTT CTC CAA TGA ATG TA' 3 and the probe used a different dye (KJD GP) 5' ROX AGG AGG CAA TAG CAT CAC TAC

ATC AA BHQ-2 3' (Eurogentec, Integrated Sciences Pty Ltd, Sydney). This assay was designed to amplify a product of 113 bp. During the initial design, the primer and probe set for each target were selected on possession of similar optimal annealing temperatures needed for the duplex assay to work effectively.

```
1   AGCTCGAGAG AGATCTCTGA AATCAACGTC TCGAAATCCA TCTTGAATAT
51  TTGTGGACTT GGACATGGAT ACAAAAATGC GACTGGGATC ACTCGCACCT
101 AATTCGTCAA CGACATTAGG GAGGAGCCTT CTCCCTTGGT TACTCAGCGA
151 CCCTACATCT TCAATCATGT TGCACATAGC CTCTGGTCTT TGATTGTTCT
201 GAATATAAAT TGTGGTCATC GATGGTTCAC ATCCGATATA TGTTTATCTA
251 GTATTCATGT CAGCCCAAAA AAATTCTTCT AAAGTTCTCT CGCTGTTTTC
301 GTGATTATCA CCTGGGTTAT TGCTGTCCTT TATCAGTTTA ACGTTGTGGT
351 CGTACATTCT AGGAGCTCAG CTTATAATCT CGCACAAGCG TAAGACGGTA
401 CATCCATACC CCGTTTCTCG CAAGCTTCCC CCGTATCGAA GACCCCTAGA
451 TTTGATTTTA CCCTTCGCGT GGAAGATGAC CTGGCCGTTC GCGTTGTTCA
501 AAACAAGGAA TCAAGTGTGA TGTATGTAAA GCGCTCTTGT CTGGATCGCC
551 GAGTGCAACG GTATATCACA GCAATCGTCT GATAGGTTTT TCCACGCAGA
601 ACATTGCAG

KJD Duplex Assay Design
Forward Primer: 5' GGA CTT GGA CAT GGA TAC'3
Reverse Primer: 5' ACA ATC AAA GAC CAG AGG'3
Probe: 5'6-Fam CAC TCG CAC CTA ATT CGT CAA CG BHQ-1'3

LCD Assay
Forward Primer: 5' GCT TCC CCC GTA TCG AAG A'3
Reverse Primer: 5' CGA ACG ACG GCC AGG TCA TCG'3
Probe: 5' 6-FAM CCC TAG AT TGA TTT TAC CCT TCG CGT GG BHQ-1'3
```

Figure 2. 1: Sequence for the *B. cinerea* ITS region showing the position of both the LCD and KJD primers for qPCR assay (accession number AJ539088 on NCBI) (Rigotti *et al*. 2002). The positions of the primers and probes are underlined in the sequence. Black underlined areas are the primers for the new assay; red underline marks show the position of the associated probe. Blue underline indicates the position of primers for the assay designed by Cadle-Davidson (2008) and the associated probe shown in italics. Actual sequences of primers and probes are shown below the sequence.

```
5819500 CAG GTA ATG AAA TTT GAT GAC CTG AAA GAA CTT GGT AGT G
5819540 AGG GGG CTG TTA AGG TAT ATG CTT TAA ACT AAT ATG TCA T
5819590 TAA TAT TTT TCT TGG TCT TGA TGT AGT GAT GCT ATT GCC T
5819630 CCT GGT TAA ATA TAT ACA TTC ATT GGA GAA AGT AAT TAA G
5819670 AAA GTT GTT ATC CAG TGC TGA CCT CCC CAA AGT AGG TTT C
5819710 T

Forward Primer: 5'GGC TGT TAA GGT ATA TGC T'3
Reverse Primer: 5'AAT TAC TTT CTC CAA TGA ATG TA' 3
Probe: 5'ROX AGG AGG CAA TAG CAT CAC TAC ATC AA BHQ-2 3'
```

Figure 2.2: Partial sequence of the *V. vinifera* Chromosome 10 showing the position of the sequences used to design the control in the duplex assay. Refer to the NCBI website for full sequence (NC_12016.2) (Jaillon *et al*. 2007). Blue font indicates the position of the forward primer, and black bold underlined font shows the position of the reverse primer. Red font indicates the position of the probe. Sequences of primers and probe shown below the *V. vinifera* sequence

2.2.5.1. Optimisation of cycling conditions for the duplex qPCR assay

After initial testing of the new duplex assay, a gradient PCR was set up to determine the optimal annealing temperature for the new assay due to the initial PCR cycling conditions being ineffective. A total of six PCR reactions were completed to test the assay with and without the probes, using *B. cinerea* DNA, *B. cinerea* DNA diluted with Pinot Meunier DNA, *V. vinifera* cv Chardonnay DNA, *V. vinifera* cv Pinot Meunier DNA and sterile Milli Q water. Each reaction contained a total volume of 10 µL, consisting of 5 µL of 2× Qiagen Hot StarTaq *Plus* PCR master mix (Qiagen), 0.1 µM of each primer (either KJD BcF and BcR or KJD GF and GR), where applicable 0.5 µM of probe (either KJD BcP or KJD GP), 2 µL of sample. The sample was either 1) *B. cinerea* DNA; 2) *B. cinerea* DNA diluted in Pinot Meunier DNA (total amount of 5 ng); 3) 10 ng *V. vinifera* DNA (Chardonnay) or 4) sterile Milli Q water. The reaction volume was made up to 10 µL using sterile Milli Q water.

Reactions were set up in a 96 well plate, which was sealed using a sterile silicon mat. A BioRad C1000 Thermo Cycler PCR machine was used and the following cycling conditions were used as recommended by Qiagen and were as follows: an initial activation step of 95°C for 5 min followed by 35 cycles of 94°C for 1 min and a gradient annealing step set at the following temperatures of 60°C, 59.4°C, 58.3°C, 56.3°C, 52°C, 50.7°C and 50°C for 1 min and extension at 72°C for 1 min after cycling, and a final extension step of 72°C for 10 min. All reactions were then run on a 2% agarose gel (Amresco Ltd, Ohio USA) containing Gold View nucleic acid stain (Guangzhou Geneshun Biotech Ltd, China) at 80 volts for approximately 1 hour and then viewed via a gel documentation system (XR model, BioRad Pty Ltd). A quick load low molecular weight ladder (New England BioLabs, USA) was used to assess the size and amount of PCR products.

The results from the gradient PCR indicated that the optimal annealing temperature for both the *B. cinerea* and *V. vinifera* primers and probe was 50°C rather the initial 60°C that was recommended during the design process in consultation with Dr Fabrice Magnino from Integrated Sciences for use in the real-time qPCR machine. Further testing also found that an extra extension step had to be added to the cycling

conditions as analysis of the data resulting from the original two-step program had found that the probes for both assays were not binding to the template. This was because the *Taq* polymerase was not reacting efficiently at the lower temperatures, which resulted in lower reaction efficiency and lower fluorescence signals. The final cycling conditions included an initial activation step of 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 50°C for 1 min and the extra extension step of 72°C for 15 s. Fluorescence data were collected after the annealing step of 50°C for both the FAM (Green, *B. cinerea* probe) and ROX (Orange, *V. vinifera* probe) channels. All qPCR reactions were performed in the Corbett RotorGene 3000 real time qPCR machine (Qiagen Pty Ltd). All qPCR reactions contained the following: - 12.5 µL 2× StrataGene Brilliant® II qPCR Master Mix (StrataGene, Agilent Technologies, California, USA), 0.3 µM of each primer (either or both KJD BcF and BcR or KJD GF and GR), 0.2 µM of probe (KJD BcP and or KJD GP), with the variation only occurring in the volume of DNA solution where applicable and sterile Milli Q water to make up a final volume of 25 µL.

2.2.5.2. Optimisation of total DNA amount per reaction

A dilution series containing *B. cinerea* and *V. vinifera* cv Chardonnay DNA was prepared as previously described in Table 2.1. The dilution series was used to apply the duplex qPCR assay to determine the optimal amount of DNA to add to the reaction to obtain the most efficient reaction. The three amounts of total DNA per standard solution (including both *B. cinerea* and *V. vinifera* DNA, or *B. cinerea* only) were 10 ng in 2 µL, 15 ng in 3 µL and 20 ng in 4 µL. A duplicate of each DNA standard and volume was tested. All qPCR reactions contained the following: - 12.5 µL 2× StrataGene Brilliant® II qPCR Master Mix (StrataGene, Agilent Technologies, California, USA), 0.3 µM of each primer (either or both KJD BcF and BcR or KJD GF and GR), 0.2 µM of probe (KJD BcP and or KJD GP), with the variation only occurring in the volume of DNA solution where applicable and sterile Milli Q water to make up a final volume of 25 µL. The modified cycling conditions were that of which is mentioned in Section 2.2.5.1.

The qPCR reactions were then run on a 2% agarose gel (Amresco Ltd, Ohio USA) containing Gold View nucleic acid stain (Guangzhou Geneshun Biotech Ltd, China)

at 80 volts for approximately 1 hour and then viewed via a gel doc (XR model, BioRad Pty Ltd). A quick load low molecular weight ladder (New England BioLabs, USA) was used to assess the size and amount of the PCR products. Band intensity was compared along with the qPCR data generated from the run (Ct values, slope and reaction efficiency) for each of the volumes, to determine the optimal volume of DNA sample to add to each reaction.

2.2.5.3. Optimisation of V. vinifera DNA amount in standard dilution series

Initial testing found that the concentration of the *V. vinifera* DNA used for the dilution series presented in Table 2.1 interfered with the reaction for the detection of *V. vinifera* and *B. cinerea* DNA. Preliminary tests indicated that the concentration of 5 ng/µL for the *V. vinifera* DNA was having an inhibitory effect on the assay's ability to efficiently detect the *B. cinerea* DNA within the samples. A qPCR was set-up to determine the optimal concentration of the grape DNA solution that would be used in creating a dilution series to obtain the standard curve to be used in quantifying the amount of *B. cinerea* DNA in samples. The *V. vinifera* DNA (5 ng/µL) stock, used in the previous experiments to create the dilutions series, was diluted 10X (final concentration of 0.5 ng/µL), 20× (0.25 ng/µL), 30× (0.166 ng/µL), 40× (0.125 ng/µL) and 50× (0.1 ng/µL). The diluted *V. vinifera* stocks were then used as a dilutent to set-up a fresh dilution series with the *B. cinerea* stock (5 ng/µL) (refer to Table 2.1 for original dilution series and concentrations of *B. cinerea* in each standard). Standards 1 and 6 (Table 2.1) with a final concentration of *B. cinerea* DNA of 2.5 and 0.0008 ng/µL were used for each of the dilution series. Reaction components are listed in Section 2.2.5.2 with both the Botrytis primers and probe set and the *V. vinifera* primer and probe set used in each reaction tube, with 2.5 µL DNA per reaction. Once the optimal dilution factor was determined, a new dilution series was prepared (Table 2.2) and tested against the original dilution series (Table 2.1) to determine the effect on the Ct values and reaction efficiency for the quantification of both *B. cinerea* and *V. vinifera* DNA. The cycling conditions used are described in Section 2.2.5.1.

Table 2.2: The concentration of *B. cinerea* DNA (stock at 5 ng/µL) and *V. vinifera* DNA (stock at 0.2 ng/µL) for the new dilution series.

Standard	DNA concentration(ng/µL)	
	B. cinerea	*V. vinifera*
Botrytis	5	-
1	2.5	0.18
2	0.5	0.18
3	0.1	0.196
4	0.02	0.1002
5	0.004	0.19984
6	0.0008	0.199968
7	0.00016	0.1999936
Grape	-	0.2

2.2.5.4. Detection limit of grape assay

The detection limit for *V. vinifera* DNA was determined by qPCR using only the grape DNA probes and primers. Two overlapping dilution series of *V. vinifera* cv Chardonnay DNA were made up using a stock solution of 5 ng/µL and 0.2 ng/µL with sterile Milli Q water as the diluent (refer to Table 2.3 for concentration of dilution series). As the optimal concentration of *V. vinifera* DNA had been determined to be 0.2 ng/µL for the stock, it was decided to test the dilution series against the higher concentrated stock used in the early optimisation, to firstly see if the higher concentration caused inhibition and to determine the limit of detection of *V. vinifera* DNA for the assay. Each dilution series was then tested in duplicate using the primers and probe designed for the internal control (detection of *V. vinifera* DNA in sample). Each reaction contained components listed in Section 2.2.5.2, with only the *V. vinifera* primer and probe set being used (KJD GF, KJD GR and KJD GP) with 2.5 µL of DNA sample. Each DNA sample was tested in duplicate. Cycling conditions were those detailed in Section 2.2.5.1.

Table 2.3: Dilution Series Concentration of each standard using the two *V. vinifera* stock solutions (5 ng/µL and 0.2 ng/µL).

Dilution Series using 5 ng/µL *V. vinifera* DNA		Dilution Series using 0.2 ng/µL *V. vinifera* DNA	
Standard	Final Concentration (ng/µL)	Standard	Final Concentration (ng/µL)
Stock	5	Stock	0.2
1	2.5	1	0.1
2	0.5	2	0.02
3	0.1	3	0.004
4	0.02	4	0.0008
5	0.004	5	0.00016
6	0.0008	6	0.000032
7	0.00016	7	0.0000064

2.2.5.5. Effect of diluent in the standard dilution series

The effect that *V. vinifera* DNA as a diluent in the *B. cinerea* dilution series relative to water as a diluent had on the reaction efficiency and Ct values of the qPCR assay was examined. A dilution series containing *B. cinerea* DNA and sterile Milli Q water as a diluent was prepared (refer to Table 2.1 for the concentrations of *B. cinerea* DNA for each standard). After optimisation for the stock concentration for the *V. vinifera* DNA, a second dilution series was prepared for the quantification of *B. cinerea* in field samples (refer to Table 2.2 for dilution series and Section 2.3.2.3 for the results of the optimisation of the *V. vinifera* amount). Each standard in both dilution series was tested in duplicate. Simplex reactions were set up for detecting *B. cinerea* DNA only. Each qPCR reaction contained components listed in Section 2.2.5.2, except only the primers and probe for *B. cinerea* detection were used (KJD BcF, BcR and BcP). Each DNA standard for each of the dilution series was tested in triplicate. Cycling conditions were those detailed in Section 2.2.5.1.

2.2.5.6. Comparison of simples and duplex qPCR reactions

An experiment was conducted to determine the effect of a duplex reaction on the reaction efficiency and its Ct values, in comparison to a simplex reaction. The dilution series used to compare the two styles of reaction set-up was that shown in Table 2.2. For the simplex reaction only, the primers and probe for the detection of *B. cinerea* was used (KJD BcF, BcR and BcP), and the reaction components were those detailed in Section 2.2.5.2, excluding the KJD GF, GR and GP primers. The duplex

reactions set-up used the same concentration of primers and amount of mix of detailed in Section 2.2.5.2. The total volume of each the simplex and duplex reactions was 25 µL, which was adjusted using sterile Milli Q water accordingly. Both the simplex and duplex reaction design were tested in duplicate for the dilution series used. Refer to Section 2.2.5.3 for the qPCR cycling conditions.

2.2.6. Testing of field samples

Fourteen grape samples out of a collection that were taken during the 2007- 08 season from a small plot trial were used to test the duplex assay (refer to Chapter Four for details of trial). Each grape sample consisted of 12 berries, that were randomly selected from 12 tagged bunches on 08/04/2008 spread across two vines. DNA was extracted in 2008/09 as described in Sections 2.2.2. All DNA samples were stored in a -20°C freezer until analysed. Samples that were selected were those from the harvest stage with 13 samples from replicated plots that had not been treated with fungicide and one (sample 2) from a treatment that had been subjected to a mid-season spray. The components of each qPCR reaction that used 2.5 µL of DNA solution are detailed in Section 2.2.5.2. Samples were tested in duplicate using the cycling conditions as described in Section 2.2.5.1.

2.2.7. Data analysis

Data were collected using the RotorGene Software supplied with the PCR machines, while analysis of results was completed using a later version of the software Rotor-Gene Q, Pure Detection (version 1.7, Build 94). Data sorting and basic analysis was done using Microsoft Office Excel (Mac 2008, version 12.2.7). Reaction efficiency (E) was calculated according to the equation reported by Bustin *et al*. (2009): (Equation B2, Appendix B). Detailed statistical analysis involving linear regressions was completed using GenStat 10th Edition. A multiple general linear regression analysis was used to compare the standard curves to determine if there were any significant differences between them. Standard errors (SE) were also calculated for Ct values when reactions were run in triplicate.

2.3. Results

2.3.1. Adoption and optimisation of technique

2.3.1.1. DNA extraction

DNA yield from B. cinerea and V. vinifera leaves

According to the Pico Green assay, DNA extracted from the bulk isolate of *B. cinerea* resulted in DNA concentrations ranging from 60 to 96 ng/µL. The variation of the yield may have resulted from the amount of mycelia transferred to Eppendorf and the extra cleaning steps removing some DNA in the samples. For the *V. vinifera* leaves (Pinot Meunier, Riesling and Chardonnay), the DNA concentration ranged from 22 to 100 ng/µL.

Field samples

The concentration of DNA extracted from the grape berry samples ranged from amounts that could not be quantified via the Pico Green assay to 30 ng/µL, with most samples having a concentration near 5 ng/µL.

2.3.1.2. Optimisation of Taqman® assay

For samples containing 10 ng *B. cinerea* DNA, the size of the PCR product using the LCD primer and probe set was approximately the expected size of 67 bp, as it was less than 100 bp represented by the lowest band in the DNA size ladder (Figure 2.3). As the annealing temperatures increased above 58.3°C there was a decrease in the amount of PCR product produced; therefore, an annealing temperature of 58.3°C was selected for all future qPCR reactions using this primer and probe set. This temperature was lower than the 60°C used by Cadle-Davidson (2008). A temperature lower than 58.3°C was not selected as it may have reduced the efficiency of the primers and probe to bind to the target during the qPCR, as the cycling is a two-step program that includes combined annealing and extension steps. In contrast, traditional PCRs have separate annealing and extension steps. Further lowering of the temperature may have also increased the risk of reduced specificity of the primers and probe, leading to increased fluorescence.

Analysis of the PCRs using Pinot Meunier DNA and sterile water controls, using an annealing temperature of 58.3°C, revealed that there was no contamination by *B. cinerea* (figures not shown), or non-specific binding of the primers and probe based on examination of the gel after electrophoresis.

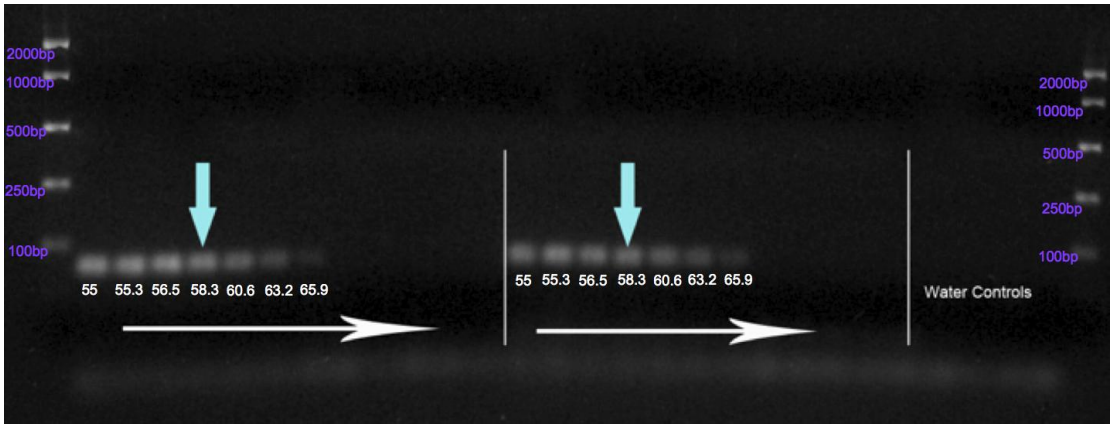

Figure 2.3: PCR results using the primers and probe from the LCD qPCR assay and 10 ng *B. cinerea* DNA: DNA size ladder at either ends of the gel (Bioline EasyLadder 1) with the base pairs (bp) shown below the corresponding band (in purple). The horizontal white arrows signify annealing temperature increasing from 55°C to 75.4°C. Where there was a reaction, the actual annealing temperature for that reaction is shown below the band. First series of samples up to the first vertical line are reactions with primers only; second series are reactions containing primers and probe. The PCR reaction with the optimal annealing temperature of 58.3°C is highlighted by aqua arrow pointing to the DNA band.

2.3.1.3. Reaction mix comparison

The qPCR results indicated that false positives occurred whenever *B. cinerea* DNA was frequently detected in water controls using either of the qPCR reaction mixes tested (refer to Tables 2.4 & 2.5). For samples containing *B. cinerea* DNA only, results from the Qiagen mix were similar across the 3 days (Table 2.5) relative to those using Bioline SensiMix dT (Table 2.4), where the Ct values ranged from 23.86 (Day Two) to 28.39 (Day 1). Bioline's reaction mix (SensiMix dT) gave Ct values for the water controls on each day that were dissimilar among runs over three consecutive days. Overall they ranged from 26.63 to 39.40 (refer to Table 2.4). All water controls tested using the reaction mix from Qiagen (Rotor-Gene Probe PCR mix) produced Ct values, except on Day 3, when only one water sample produced a Ct value. Overall, these water samples all produced Ct values in the last three cycles (37-40), which, depending on the Ct value for the

lowest standard suggested that there was some background interference with fluorescence detection (refer to Table 2.5).

Reactions containing *B. cinerea* DNA produced a band of 67 base pairs when visualised using gel electrophoresis (figures not shown). No such product was observed for the water controls using the Bioline reaction mixture, where the sample appeared to be retained in the wells. As the supply of Qiagen reaction mix was limited, testing grape DNA and other *B. cinerea* standards was not attempted. Nevertheless, it appeared that the Qiagen mix performed better as *B. cinerea* DNA was not amplified for two of the samples on day three and on other days the water controls all gave higher Ct values, as opposed to the Bioline mix which gave similar Ct values closest to the lowest standard (concentration 0.004 ng) (refer to Tables 2.4 and 2.5). Overall, the assay proved to be unreliable for the PCR machine and laboratory conditions under which these probes and primers were tested.

Table 2.4: Cycle Threshold (Ct) values generated using the qPCR mix Bioline SensiMix dT®. Assays were conducted over 3 consecutive days using 3 different water sources. Samples were tested in either duplicate or quadruple.

Amount of DNA (ng) per reaction			Cycle Threshold (Ct)		
B. cinerea	*V. vinifera*	Cultivar	Day 1	Day 2	Day 3
25	0		28.39	23.86	27.18
12.5	12.5	Pinot Meunier [a]	29.12 28.41	24.66 24.72	26.18 28.10
0.5	24.5	Pinot Meunier [a]	34.61 34.62	30.64 30.48	26.11 28.03
0.004	24.996	Pinot Meunier [a]	37.31 32.72	32.80 33.39	28.08 28.62
0	25	Pinot Meunier [a]	35.55 35.75	28.76 31.03	35.61 30.51
0	25	Riesling [b]	36.03 36.43	32.42 31.46	32.59 29.55
H_2O [c]			36.49 33.66 36.91 36.63	32.93 33.71 33.84 34.29	39.20 34.17 27.08 26.63

[a] sourced from micropropagated vines
[b] sourced from glasshouse vines
[c] Day 1 H_2O = Qiagen's RNASE Free H_2O; Day 2 H_2O= autoclaved Milli Q H_2O; Day 3 H_2O= autoclaved and filter sterilised Milli Q H_2O

Table 2.5: Cycle Threshold (Ct) values generated using Qiagen's Rotor-Gene Probe PCR mixes. Assays were conducted over 3 consecutive days using a different water source for each day. Multiple values of Ct for the reaction containing water only represent triplicate samples.

Sample Type	Amount of DNA	Cycle Threshold (Ct)		
		Day 1	Day 2	Day 3
B. cinerea	25	27.11	26.36	27.04
Water [b]	-	37.93	38.41	dna [a]
		39.61	39.20	38.97
		38.62	38.32	dna [a]

[a] dna= did not amplify.
[b] Day 1 H_2O = Qiagen's RNASE Free H_2O;
Day 2 H_2O= autoclaved Milli Q H_2O;
Day 3 H_2O= autoclaved and filter sterile Milli Q H_2O

2.3.2. Re-designing the qPCR – a duplex reaction

As previously stated in Section 2.3.1 it was concluded that the assay originally designed by Cadle-Davidson (2008) would not be suitable for further study due to the background fluorescence occurring in the water controls. The simplex assay also did not take into consideration the effect that PCR inhibitors might have on the reaction efficiency if applied to field samples, or ensure that the negative results were valid. All of these factors resulted in initiating the design process for the new duplex Taqman® assay that would be used for further study (refer to Section 2.2.5 for further detail).

2.3.2.1. Optimisation of cycling conditions - optimal annealing temperature

As previously stated, the optimal annealing temperature determined by the gradient PCR for the new assay (*B. cinerea* and grape) was 50°C. There was no PCR product found when the initial recommended annealing temperature for both sets of primers and probes was 60°C with no band present after gel electrophoresis (Figures 2.4, 2.5 and 2.6). The PCR reactions using the primer and probe set appeared to have failed resulting in no product band. This may have been due to water being added instead of the *B. cinerea* stock (Figure 2.4). The gel electrophoresis results for the *B. cinerea* DNA diluted in Pinot Meunier DNA highlighted the increased sensitivity of the probe where the band intensity was significantly higher when the probe was included in the mix using lower temperatures of 56.3°C, 53.9°C, 52°C, 50.7°C and 50°C (Figure 2.5). There were

no PCR products when grape primers and probe (KJD GF, GR and GP) were tested using DNA from tissue cultured Pinot Meunier grapevines (Figure 2.6). However, PCR products were observed when Chardonnay DNA was used with the grape primers (Figure 2.6). The size of the PCR product for *B. cinerea* was 150 bp and it was 113 bp for *V. vinifera*.

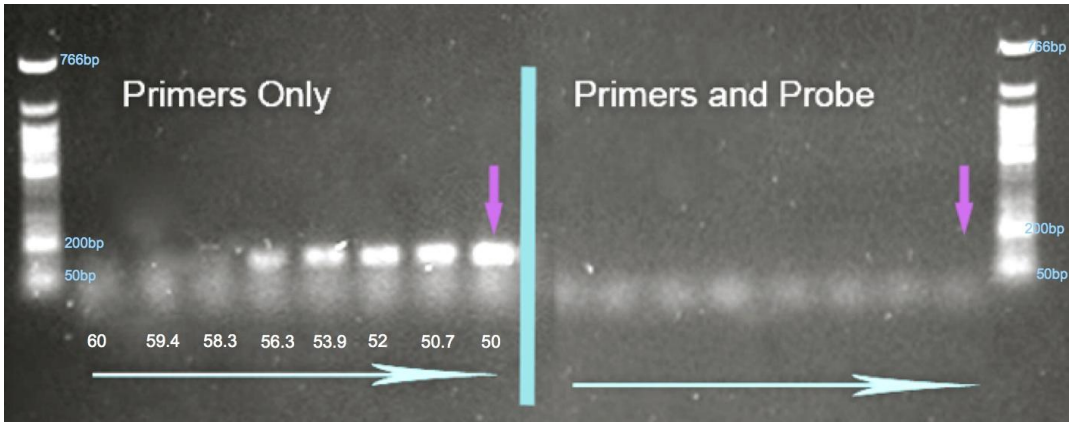

Figure 2. 4: Results from gradient PCR for new assay development showing *B. cinerea* (10 ng) DNA reacting with either primers only or with the probe (separated by vertical aqua line). Gradient temperature decreases from left to right (60°C to 50°C). Purple arrow points to the band associated with the optimal annealing temperature selected. DNA ladder at each end is Biolab's Quick Load Low Molecular Weight DNA ladder (a selection of base pairs (bp) are marked beside the corresponding band in the ladder).

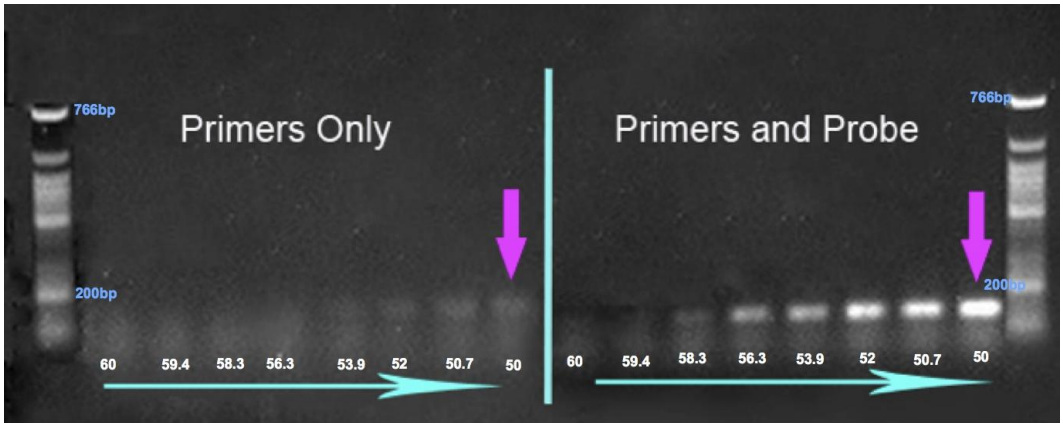

Figure 2. 5: Gel showing gradient PCR results for the new primers and Probe detecting *B. cinerea*. DNA sample used was *B. cinerea* DNA diluted in Pinot Meunier DNA in a 1:1 ratio (5 ng of each DNA). Primers only and Primers and Probe separated by vertical aqua line. Gradient temperatures decrease from left to right (60°C to 50°C). Magenta arrow points to the band associated with the optimal annealing temperature selected (50°C). DNA ladder at each end is Biolab's Quick Load Low Molecular Weight DNA ladder (a selection of base pairs (bp) are marked beside the corresponding band in the ladder).

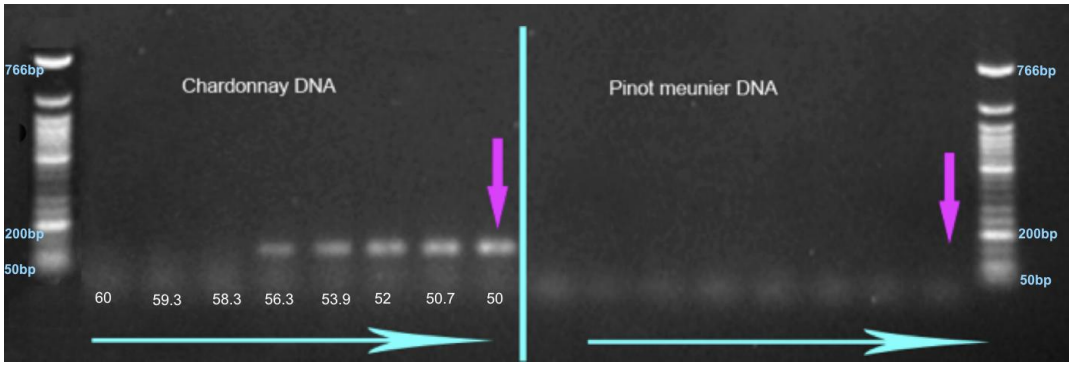

Figure 2. 6: Gel showing the gradient PCR results for the primers and probe KJD GF, GR and GP for the detection of grape DNA. Samples tested consisted of 10 ng of *V. vinifera* DNA (cv Chardonnay) or Pinot meuniere DNA (from micropropagated plants). Gradient decreases from left to right as directed by the horizontal aqua arrow (60ºC- 50ºC), with the corresponding temperatures below each band. Red arrow points to the band associated with the optimal annealing temperature of 50ºC. The DNA ladder at each end is BioLab's Quick load low molecular weight DNA ladder (a selection of base pairs (bp) are marked beside the corresponding band in the ladder).

2.3.2.2. Optimisation of DNA amount per reaction for the duplex reaction

Overall the qPCR results for each dilution series using different volumes of DNA solution were similar (refer to Figure 2.7 and Table C1, Appendix C). The regression analysis for the 2 µL volume solution resulted in a slope of 3.611 and a reaction efficiency of 89%. The 3 µL volume had a slope of 3.681 with a reaction efficiency of 87% and the 4 µL volume resulted in a slope of 3.781 and a reaction efficiency of 84% (Figure 2.7). There was no statistical difference between the linear regression lines produced from the three different volumes of DNA solution. However, there appeared to be a trend for increasing volume of DNA solution used per reaction resulting in a higher Ct value for the standard with the lowest concentration (*B. cinerea* concentration of 0.00016 ng/µL). This appeared to produce a lower PCR efficiency. Band intensity on the gel (Figure 2.8) varied for reactions containing different amounts of DNA. There appeared to be greater band intensity for the samples with the lowest concentrations of *B. cinerea* when 2 and 3 µL was used as opposed to 4 µL (Figure 2.8). The intensity of the bands for the standards with the higher amounts of DNA was found to be similar with only a slight variation in intensity (Figure 2.8). Therefore, it was decided that a volume between 2 and 3 µL would be optimal.

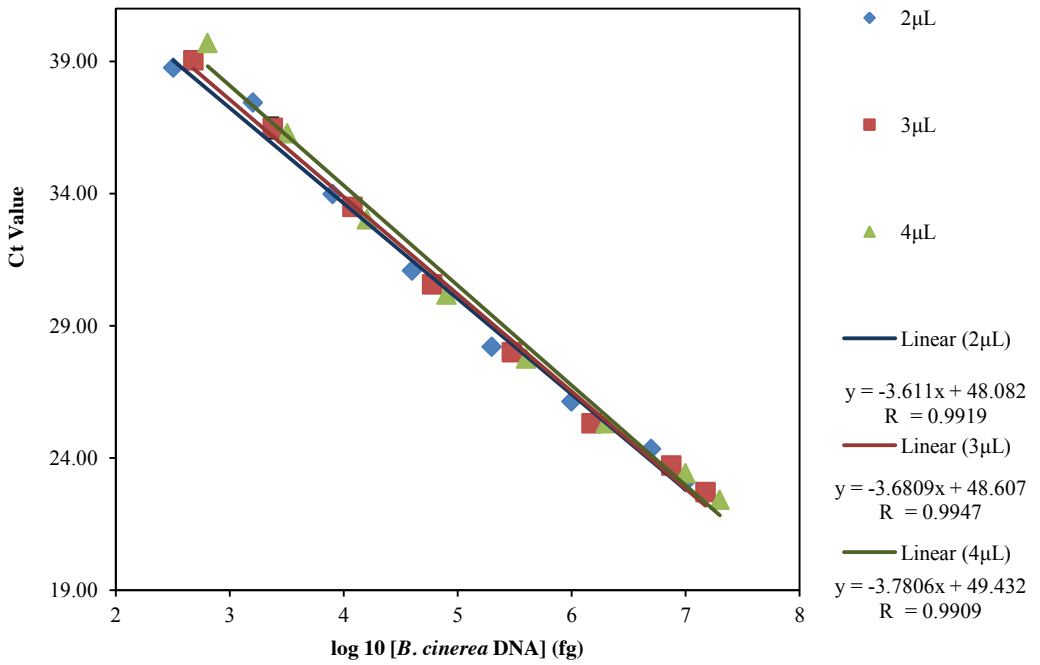

Figure 2. 7: Graph showing the relationship between volumes of DNA solutions used and mean Ct value for the Botrytis Standard dilution series using 2 µL, 3 µL or 4 µL of DNA solution. Each standard also contained *V. vinifera* DNA, which increased as the *B. cinerea* concentration decreased. The slope, intercept and R^2 value are shown for each dilution series.

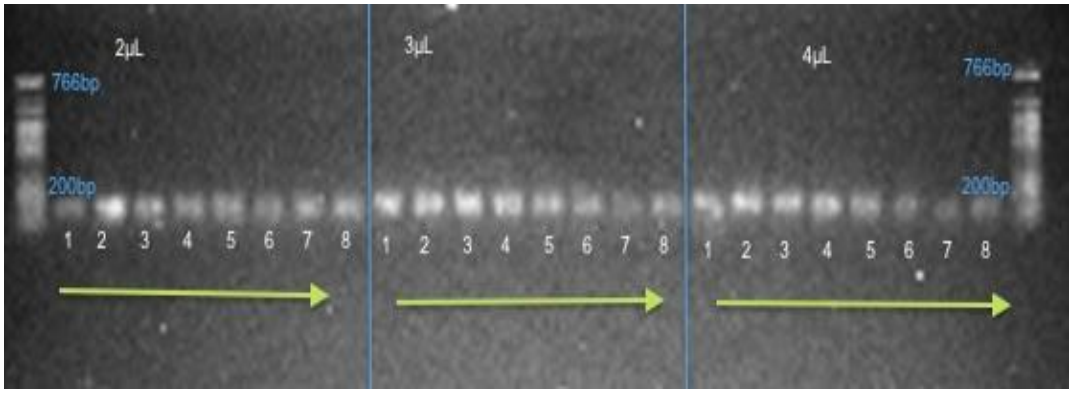

Figure 2. 8: Gel showing the differing band intensities between the different volumes of DNA used per reaction for each of the samples used in the standard dilution series separated by a line. Concentration of *B. cinerea* DNA decreases from left to right for each set of standards as indicated by aqua arrows. The concentrations of each standard is as follows: - 1) 5 ng/ µL; 2) 2.5 ng/µL; 3) 0.5 ng/µL; 4) 0.1 ng/µL; 5) 0.02 ng/µL 6) 0.004 ng/µL; 7) 0.0008 ng/µL; 8) 0.00016 ng/µL. At either end of the lanes are BioLabs Quick Load low molecular weight DNA ladder with the 200bp and 766bp band marked.

2.3.2.3. Optimisation of V. vinifera DNA amount used in dilution series preparation

A reaction prepared from the 5 ng/µL stock of *V. vinifera* (cv. Chardonnay) DNA was found to reduce the efficiency of detection of *B. cinerea* DNA, as shown by the suboptimal Ct values (30+) obtained in preliminary testing (Table 2.6). The results from the dilution analysis indicated that the optimal dilution factor of the 5 ng/µL for the stock solution of *V. vinifera* DNA was between 20× and 30× as Ct values were below 30 for the detection of *B. cinerea* in each of the standards, with similar results for the *V. vinifera* control (Table 2.6). Using a dilution factor of 40× and higher suggested that this would be too dilute for the assay to detect *V. vinifera* DNA efficiently given that no DNA was detected in the reaction using a dilution factor of 50× (Table 2.6). Overall, the best dilution factor was between 20× and 30× with optimal Ct values for detection of both *B. cinerea* and *V. vinifera* DNA. The second test using a 25× dilution factor, which corresponds to 0.2 ng/µL stock solution of *V. vinifera* DNA, was found to be optimal for the assay to detect both the *B. cinerea* DNA and the *V. vinifera* DNA for the standard dilution series (Figure 2.9; refer to Table C2 Appendix C). The line (standard curve) predicted from linear regression was found to have a slope of -3.7559, which resulted in a reaction efficiency of 85% (Figure 2.9). This dilution series was used to generate all further standard curves for quantification of *B. cinerea* DNA in field samples.

Table 2.6: Ct values obtained for the different amounts of the *V. vinifera* DNA used in the duplex reaction. The concentration of the stock solution of *V. vinifera* DNA prior to dilution was 5 ng/μL. Each diluted solution was then used to make fresh dilution series (concentrations shown below the dilutions). Ct values shown are for the detection of *B. cinerea* or *V. vinifera* DNA.

B. cinerea DNA total ng	Ct values for the detection of *B. cinerea* in standard solutions with different amounts (ng) of *V. vinifera* DNA						Ct values for the detection of *V. vinifera* DNA in standard solutions with different amounts (ng) of *V. vinifera* DNA					
Dilutions	0 ×	10×	20×	30×	40×	50×	0 ×	10×	20×	30×	40×	50×
Concentration	5 ng/μL	0.5 ng/μL	0.25 ng/μL	0.167 ng/μL	0.125 ng/μL	0.100 ng/μL	5 ng/μL	0.5 ng/μL	0.25 ng/μL	0.167 ng/μL	0.125ng/μL	0.10 ng/μL
0.25	28.05	27.42	27.68	29.02	25.75	25.01	25.57	26.07	25.83	25.99	30.95	0
0.002	28.35	31.41	29.77	28.83	28.55	-	25.65	28.65	27	30.31	39.69	0

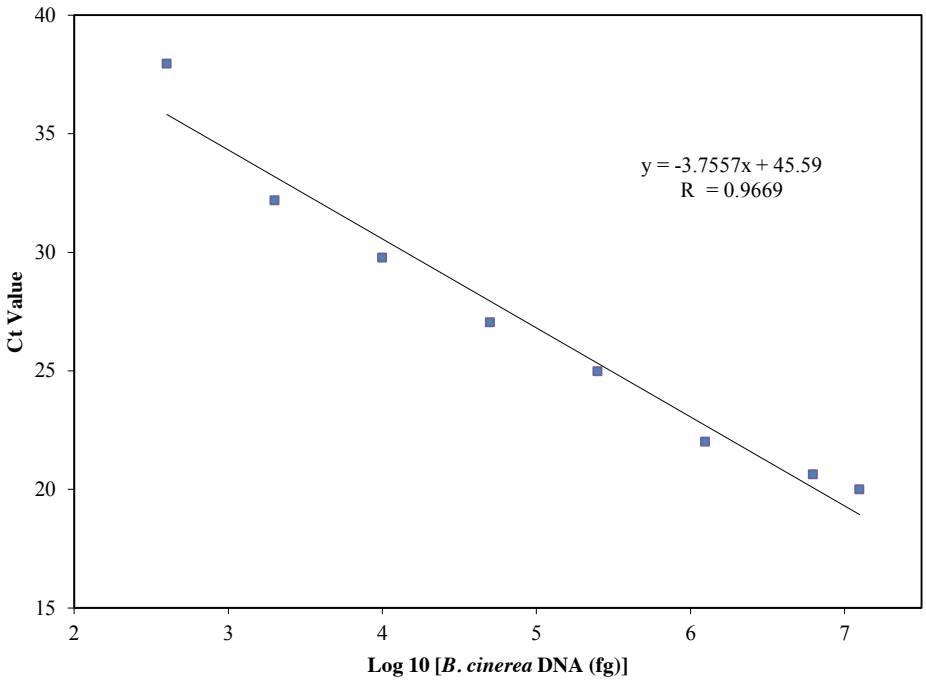

Figure 2. 9: Standard curve for the optimised dilution series using 0.2 ng/µL *V. vinifera* stock as diluent. Mean Ct values are for the *B. cinerea* DNA. The slope, intercept and R^2 value of the line predicted from linear regression is also presented.

2.3.2.4. Detection limit of grape assay

A comparison between diluting the concentrated stock of 5 ng/µL and the 0.2 ng/µL stock solution revealed that high amounts of DNA were inhibitory to the reaction for the detection of *V. vinifera* DNA (Figure 2.10, Table C3, Appendix C). The assay was able to detect as little as 16 fg of *V. vinifera* DNA, highlighting its sensitivity (refer to Table C3, Appendix C). The reaction efficiency for the dilution series, when using the 5 ng/ µL stock solution of *V. vinifera* DNA, was calculated to be 113 %, which reflected the calculated slope value of -3.043 and lack of detection of *V. vinifera* DNA in two of the standard solutions (Figure 2.10). The reaction efficiency for the dilution series using the 0.2 ng/µL stock was calculated to be 167 % with a calculated slope of -2.342 (Figure 2.10).

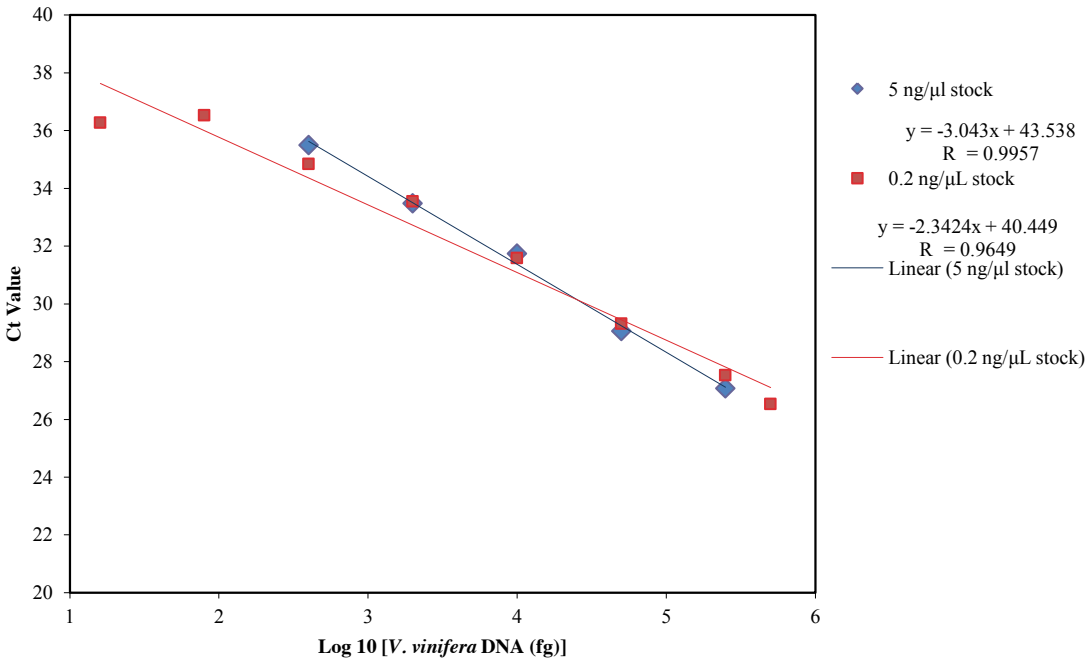

Figure 2. 10: Linear regression of mean Ct values (duplicate samples) for the two standard dilution series to test the detection limit for *V. vinifera* DNA. Two regression lines are shown 1) 5 ng/µL stock used to make dilution series 1 and 2) the 0.2 ng/µL stock used to make the new dilution series. The slope, intercept and R^2 value are also shown for each of the regression line produced for the dilution series.

2.3.2.5. Effect of diluent in the standard dilution series

Using water as a diluent tended to result in earlier amplification than using *V. vinifera* DNA (Figure 2.11; refer to Table C4, Appendix C), although the reaction sensitivity for the dilution series was similar (Figure 2.11). The dilution series using water had a higher R^2 value (0.98) and a higher slope value (- 3.308) indicating that the reaction efficiency was 100 %, while the series using *V. vinifera* had a lower R^2 value (0.95) and had a lower slope value (- 3.091) indicating a reaction efficiency of 110 % (Figure 2.11). However, statistical analysis via linear regression analysis (curves generated) of the two dilution series found they were not statistically different (P = 0.537).

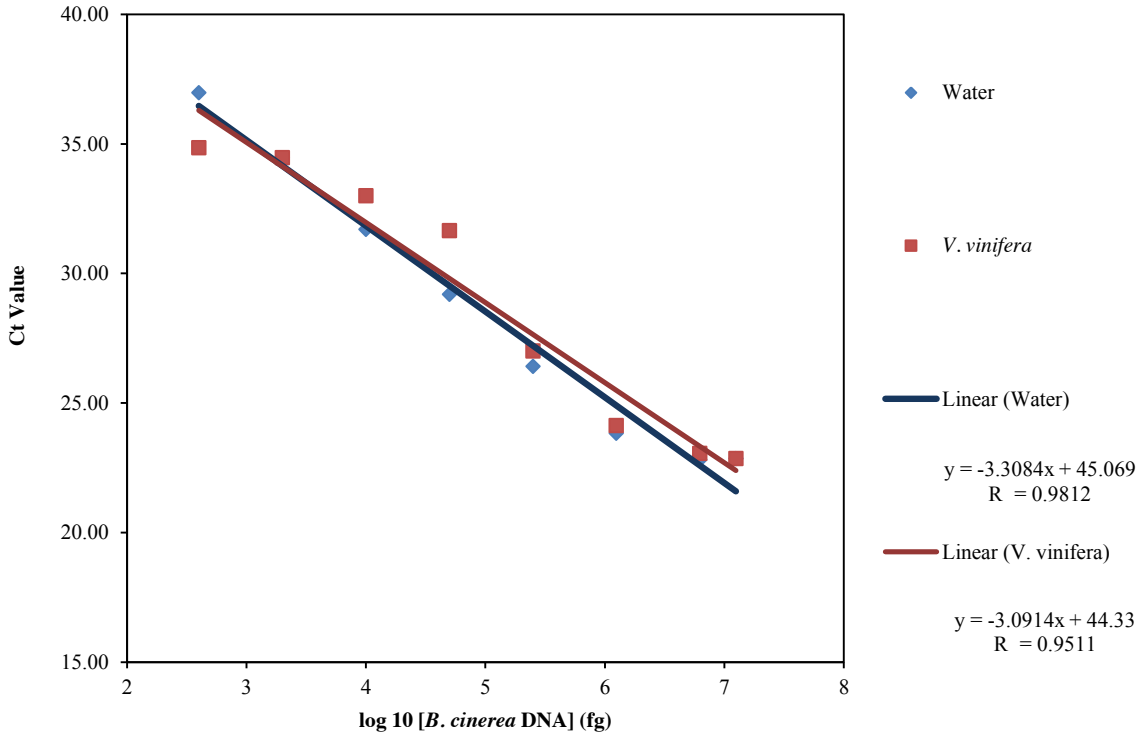

Figure 2. 11: Linear regression showing the mean Ct values for *B. cinerea* DNA solution diluted in *V. vinifera* cv Chardonnay DNA solution versus *B. cinerea* DNA solution diluted in water. Linear equation with R^2 values is also shown for each of the standard curves.

2.3.2.6. Comparison of simplex and duplex qPCR reactions

There were slight differences between the simplex and duplex reactions, mainly for reaction efficiency (Figure 2.12). The duplex reaction was found to be less efficient with an efficiency of 84 % and a slope for the standard curve of -3.7911. The simplex assay had a reaction efficiency of 110 % with a slope for the standard curve of -3.090 (Figure 2.12). Comparison of the Ct values showed that there were larger differences between equivalent standards when the *B. cinerea* DNA amounts were lower, especially below 0.01 ng (Table C5, Appendix C). The R^2 values for both linear relationships were also different since the duplex assay had a higher value of 0.98 and the simplex assay had a value of 0.95 (Figure 2.12). Statistical analysis showed that there was no significant difference between the two linear regression lines ($P = 0.08$).

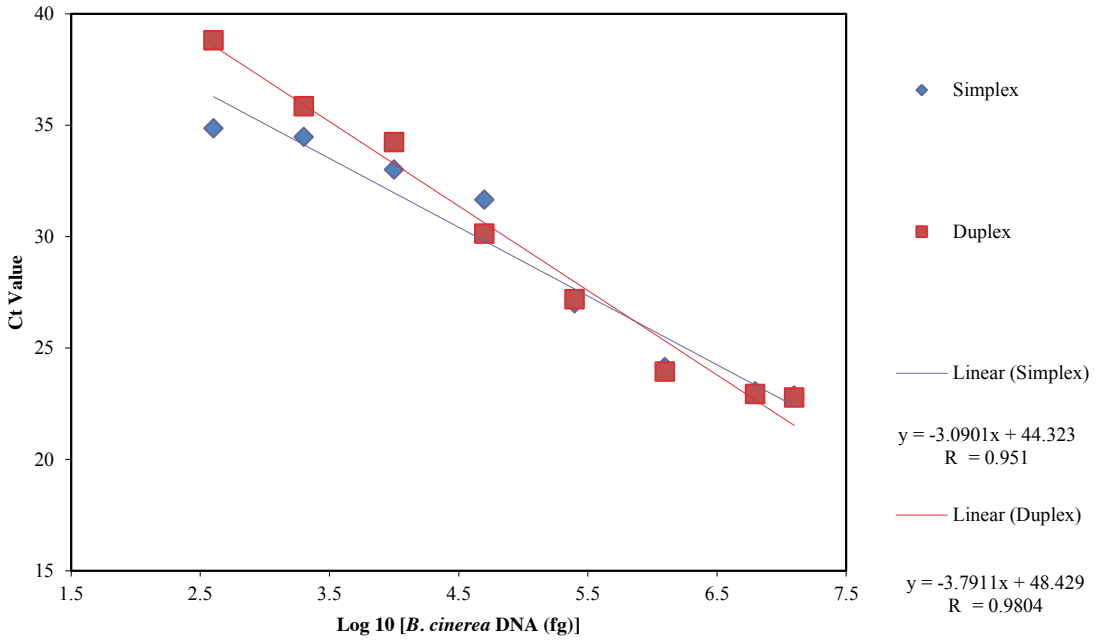

Figure 2. 12: Linear regression for the dilution series tested as a duplex reaction (amplifying *B. cinerea* and *V. vinifera* DNA concurrently) compared to that of a simplex assay (amplifying *B. cinerea* DNA only). Mean Ct values are shown. R^2 values are shown or each of the dilution series along with the linear equation, y intercept and slope.

2.3.3. Testing of field samples

The R^2 value for the standard dilution series was 0.98, which is in the optimal range of between 0.95-1.0; however, reaction efficiency was 83% (Figure 2.13, Table 2.7). The duplex assay was able to detect *B. cinerea* DNA in 7 of 14 samples of grape berries from the field (Table 2.7). Only one sample (sample 12) failed to detect any DNA, which suggests that there was either no grape DNA in the sample or not enough DNA for detection. The assay was able to detect as little as 8 fg of *B. cinerea* DNA in a sample (sample 9) and the highest amount detected was 0.068 ng of DNA (sample 5) (Table 2.7). Even though there was detection of *B. cinerea* DNA in sample 9, the Ct value registered was towards the end of the cycles for PCR, which could be interpreted as a negative result. The grape assay for sample 9 was positive which suggests a valid result.

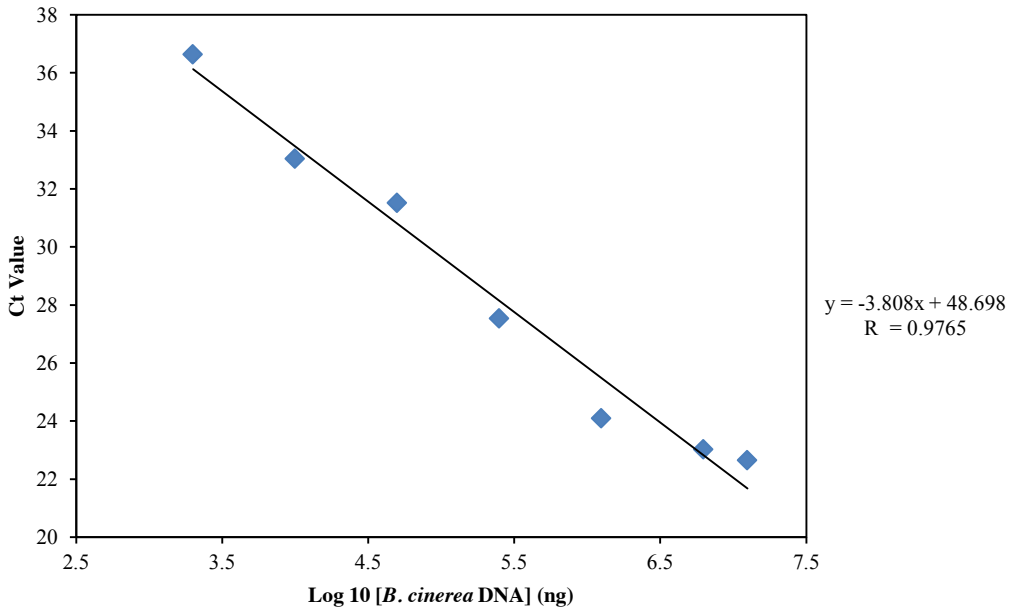

Figure 2. 13: Results of a duplex assay applied to a standard dilution series. This standard curve was used to calculate the amount of *B. cinerea* DNA in field samples. Standards were run in duplicate with the mean shown in the figure.

Table 2.7: Results of the duplex qPCR assay applied to samples of berries from *V. vinifera* cv Chardonnay grown under commercial conditions at Rokeby, Tasmania. The mean cycle threshold (Ct) value and the amount of *B. cinerea* DNA in the sample are displayed. A failed result (F) was recorded when neither *B. cinerea* or *V. vinifera* (Vv) DNA was detected.

Sample	Quantification of *Botrytis cinerea* DNA		Vv
	Ave Ct	Amount of DNA (fg)	Mean Ct
1	34.14	2.405	32.98
2	38.87	0.118	34.09
3	0	0	25.68
4	33.65	3.274	28.53
5	28.87	68.193	26.83
6	0	0	25.36
7	0	0	27.67
8	0	0	28.63
9	39.45	0.082	26.11
10	29.45	47.327	29.04
11	0	0	28.63
12	F	F	F
13	35.12	1.2887	34.52
14	0	0	35.92 (0)
	R^2	0.98	
Reaction Efficiency (%)		83	

2.4. Discussion

Adoption and optimisation of a quantitative PCR (qPCR) assay is not always a straightforward process. Prior to and during adoption of a new technique, it is important to learn about the methods encompassing it and understand the data that are generated. As highlighted here, sometimes when initial testing fails, the choice is either to abandon trying to optimise the assay to suit the new laboratory conditions or to design a new assay based around the already published assay.

The choice of reaction mix plays an integral part in determining both PCR and qPCR results, as highlighted in this study during the optimisation of the qPCR assay designed by Cadle-Davidson (2008). The real-time qPCR tested with both reaction mixtures resulted in fluorescence that presumably was from non-specific binding, as Ct values registered when both water and grape DNA were used. Moreover, results of gel electrophoresis suggested that there was no cross contamination from *B. cinerea*. Reaction mixes designed for both PCR and qPCR may vary in concentration of key components such as dNTPs, *Taq* polymerase and magnesium chloride concentration, as well as company-specific modifications that are not specified in the data sheets, all of which could result in the varying interactions between the primers, probe and DNA sample. Any of these might influence the reaction efficiency, primer and probe binding to the target, or primer-dimer formation. There are at least three potential reasons for failure of the assay. First, there was a greater risk of the production of dimers, cross dimers, and hairpins, as described previously. Second, the original assay was based around a very small section of the *B. cinerea* sequence in which there was only 1 bp on either side of the probe between the primers. Third, internal reference dyes that are used in block-style thermocyclers (used by Cadle-Davidson 2008) to measure and subtract background fluorescence are not used/required in rotor-style machines. The purpose of the dye is to compensate for the temperature variation that may occur with the PCR machine affecting the annealing of primers and probe to the target. When all three of these features are considered together, there may have been an increased risk of the probe binding onto the primers resulting in a greater risk of the machine measuring the fluorescence resulting from the non-specific binding. Non-specific binding may have been less of an issue for the block-style thermocycler used by Cadle-Davidson (2008). Rotor-

based machines do not require these additional dyes in the reaction mix because each reaction tube is subjected to the same temperature from the constant spinning, with no or little temperature variation among tubes. Given the results, and the goal of eliminating or reducing the chance of significant background fluorescence, the next step was to design a new primer and probe set.

In PCR and qPCR, it is common to include a negative control with no template DNA to ensure that there is no contamination from target DNA or non-specific amplification of DNA in the reaction solutions. A negative control can be water or DNA to which the primers and probe would not bind to during cycling. In this case, *V. vinifera* DNA was used. In reactions where there is amplification of the target DNA from negative controls, a Ct value may be acceptable if it is 3.3 Ct values above the lowest standard used (Smith and Osborn 2008). It may have been possible to continue using the assay designed by Cadle-Davidson (2008) with the Qiagen real-time probe reaction mix if Ct values for negative controls were sufficiently and consistently high. Further testing with the dilution series and field samples would be needed to check for assay reproducibility. Another consideration is that too many cycles in a qPCR may lead to an increased risk of both negative and no template (water) controls registering Ct values due to an increase of background fluorescence as the run progresses. In PCR reactions, often the number of cycles is limited to 30-40 cycles and for many real-time qPCR applications the limit is 40 cycles (Coolong *et al*. 2008; Delaherche *et al*. 2004; Dorak 2011; Selma *et al*.2008; Suarez *et al*. 2005). It is widely accepted that often anything detected after 40 cycles is a false positive, as potentially one is quantifying something that is actually not present (Bustin *et al*. 2009; Dorak 2011). Reducing to the number of cycles to $\leq$ 40 would ensure that background fluorescence that may increase over time in negative or no template controls remains below the level of detection. Otherwise, qPCR results would need to be validated by gel electrophoresis of PCR products to ensure that the fluorescence was not caused by contamination.

The next step was to design a new assay based on the *B. cinerea* sequence published by Rigotti *et al*. (2002) for use with Taqman-based chemistry, and develop a duplex reaction. Diguta *et al*. (2010) developed a duplex assay using primer sequences that were originally published by Suarez (2005) for the detection of *B. cinerea* in the grape

samples. In the study, the samples were spiked with DNA from the yeast *Yarrowia lipolytica* and corresponding primer sequences (Tessonnière *et al*. 2009) were used to create an internal control. The duplex assay reported here used *V*. *vinifera* DNA as both diluent and internal control, with primers based on the sequence reported by Jaillon *et al*. (2007). The dilution series was also prepared using *V*. *vinifera* DNA because unknown (field) samples were from grape berries containing mostly *V*. *vinifera* DNA. However, the risk of running a duplex may result in lower reaction efficiency compared to that of a simplex reaction as shown in this study. The decreased efficiency probably resulted from the fact that two reactions were taking place in the one tube (detection of both *B*. *cinerea* and *V*. *vinifera* DNA targets). The target would have been competing for reaction mix enzymes and would have less chance of meeting the reciprocate DNA sequence in the mixed DNA sample.

After the design of a qPCR or non-quantitative PCR, a gradient PCR reaction should be run to optimise the cycling conditions. The results highlight this important step during the optimisation of the duplex assay, in which the recommended cycling conditions failed to work with new assay. The cycling conditions for probe based qPCR reactions involves a two-step program with the second step a combined annealing/extension step with the temperature set to 60 °C. This ensures the *Taq* DNA polymerase reacts with the probe efficiently to ensure that the quencher situated at the 3' is released when the probe binds to the target after the extension phase of the two primers (Wilhelm and Pingould 2003). If the temperature is too low, it may cause the probe to sheer without binding to the target efficiently causing reduced signal, which would affect the Ct values (Wilhelm and Pingould 2003). Given this possibility, an additional short cycle at a higher temperature (72^{o}C) was added to the assay reported here.

During optimisation of the duplex assay, the gradient PCR results showed that DNA sourced from micro-propagated plants (Pinot Meunier) was not suitable to be used in the assay. As a result, the *V*. *vinifera* DNA was sourced from field or glasshouse grown grapevines. The reason behind the micro-propagated plants not being suited to the assay may potentially have been due to the samples being genetically different and not containing the Chromosome 10 gene, upon which the control was designed (Jaillon *et al*. 2007). However, DNA stocks of the *V*. *vinifera* cultivars Riesling and

Chardonnay resulted in positive results for the *V*. *vinifera* control (Figure 2.3C). Due to constraints of time and access to clean plant material, there was no further investigation of whether or not the *V*. *vinifera* primer sets worked using DNA from other cultivars. Further investigation would be needed to ensure that the assay would be suited for the quantification of *B*. *cinerea* in other *V*. *vinifera* varieties that are susceptible to BBR.

The use of host plant DNA as a diluent for the preparation of a dilution series has been demonstrated previously (Valsesia *et al*, 2005; Cadle Davidson, 2008). For example, Valsesia *et al*. (2005) developed a qPCR assay for the detection of *P*. *viticola* in grape samples, where they used a dilution series using DNA extracted from *V*. *vinifera* leaves. In contrast, some dilution series/standards are designed using cell number (no serial dilution), where a known number of cells of the internal control/ dilution component is added to the target sample prior to the DNA extraction process. If this is the case, the spiking agent acts as the control, however the source of the cells may not have originated from similar tissue to that of the co-extracted host plant, in which the target pathogen resides (Coolong *et al*. 2008; Diguta *et al*. 2010; Oliveira *et al*. 2002). Often these dilution series were developed without final quantification or normalisation of a stock solution after DNA extraction, relying purely on the qPCR to quantify the amount of DNA/copy number. There is also the issue of not taking into consideration the removal of DNA that may occur during the extraction and cleaning process and dilution of cells may not reflect the tissue type of the unknown sample.

In the present study, the use of *V*. *vinifera* DNA as the solution for serial dilution of *B*. *cinerea* had only a slight effect on Ct values, R^2 and reaction efficiency in comparison to water. The slight increase of Ct values for each of the standards in the dilution series using the *V*. *vinifera* DNA as the diluent may have resulted from the non-target DNA obscuring the target DNA for the primer and probe hybridization.

This study highlighted the importance of optimising the amount of total DNA used in a PCR reaction. The study showed that adding too much DNA could have an inhibitory effect. This was reflected in the gel electrophoresis results for which the band intensity was lower for 4 µL volumes versus 2 and 3 µL. As qPCR is a more sensitive technique than PCR without real-time quantification, either too much or not

enough DNA can have an inhibitory effect. Even though the dilution series for the different volumes of DNA solutions were not statistically different from each other, the decision to use a volume of 2.5 µL per DNA sample was based on the overall reaction efficiency being higher in the tests involving the DNA volumes 2 and 3 µL, particularly in samples with less target DNA (Figure 2.4, 2.5 and Table 2.6).

In assessing a linear curve generated for use in qPCR, the optimal R^2 value should be above 0.96, with the optimal value of 0.98-0.99. This study showed that the R^2 values varied between 0.95 and 0.99. The variation in the R^2 values from the linear curves for all qPCR runs in this study is most likely the result of manual pipetting. Often this form of error can be eliminated when a pipetting robot is used, as theoretically the R^2 achieved in assay would be consistent across all experiments and at least 0.98. The slope of the curve is also an important value, as it reflects how efficient the reaction was (Bustin 2004; Dorak 2010). The optimal slope for a dilution series curve, as previously stated is between 3.1 and 3.6, when this results in efficiency between 90 and 110% (Bustin 2004; Dorak 2010). The results from each of the curves showed that the efficiency varied. The results indicated that using *V. vinifera* DNA as a diluent reduced the efficiency of the reaction relative to water as a diluent. Presumably, the primers and probe would have to meet its target DNA in a solution that has a second DNA template; that is, the reaction mix components need to locate the target sequence in the mixture. The variations in the R^2, slope and reaction efficiency could also be due to the reaction components; all components are temperature sensitive and require to be kept cold once thawed to minimise degradation of enzymes as well as other components. Another aspect is that the probes, as well as being temperature sensitive, are light sensitive due to the fluorescence dyes that are bound to the sequence. All of these factors would affect the reaction even though exposure to light is minimised as much as possible.

Applying the assay to field samples is important to ensure that the assay will work on unknown samples and to determine if the method/sample preparation needs further optimisation. Out of the 14 field samples tested, only one sample failed in that no DNA was detected. The cause of this failure may have been due to the DNA extraction process not yielding enough DNA, or potentially pipette error as all reactions were set-up by hand. This study highlighted the reliability of the duplex

method, as the assay did not detect *B*. *cinerea* DNA in six samples, but was able to detect *V*. *vinifera* DNA. These results suggest either that there was no *B*. *cinerea* DNA present in these samples or that it was below the detection threshold.

The qPCR assay developed in this study was shown to be very sensitive with regard to the detectable amount of DNA for both *B*. *cinerea* and *V*. *vinifera*. Limits of detection are determined by extrapolation from the data and so any values presented provide a relative rather than an absolute measure of sensitivity. Based on the standard dilution series, the duplex assay was able to detect as little as 0.4 pg of *B*. *cinerea* DNA, which was similar to the limit of detection of 1 pg reported by Cadle-Davidson (2008). A direct comparison (within the same run) to Cadle-Davidson's assay was unable to be completed because of the differences in the cycling conditions. As little as 0.082 pg of *B*. *cinerea* DNA was detected in the field samples, whereas Cadle-Davidson's (2008) assay had a limit of 3.2 pg and the limit of the Diguta *et al*. (2010) assay was 6.3 pg. The detection limit for the *V*. *vinifera* DNA in the duplex assay was 8 fg, which indicated that a negative result for *B*. *cinerea* DNA will be valid when only a minute amount of host DNA is present. There do not appear to be any reports to date about temporal changes in the amount of DNA grape berries as they develop and change in terms of size or amount of fungal infection. Further investigation into this is warranted to accurately determine the expected range in the yield of *V*. *vinifera* DNA.

2.5. Conclusion

Development of quantitative PCR, like non-quantitative PCR, can involve many steps including adoption of an existing assay and optimisation for different equipment and/or reaction components, as well as redesigning and optimising a new assay. After the completion of the optimisation steps, the duplex qPCR assay has been shown to quantify *B*. *cinerea* DNA in grape berry samples collected from the field. This DNA was from naturally occurring infections of *B*. *cinerea*. The preliminary testing of the assay indicated that it warrants further testing as a tool for quantifying *B*. *cinerea* during epidemics of BBR.

Chapter Three
Temporal progression of *B. cinerea* over a grape growing season

3.1. Introduction

The development of any plant disease is a spatio-temporal process that is initiated when a pathogen infects the host plant. *Botrytis cinerea* has the ability to infect the grape bunch at multiple stages from flowering onwards, with disease symptoms only becoming visible during the later stages of ripening when it is often too late to implement control measures (Elmer and Michailides 2004). Temporal progression curves produced for plant diseases can help to understand the complex relationship between the pathogen and its host, in this case *B. cinerea* and the grapevine. They also can be useful tools in predicting potential yield loss and quality downgrades, and can help inform management decisions that have to be made to reduce the disease risk (Jeger 2004). Currently there are numerous techniques used to develop disease progress curves for monitoring and quantifying plant diseases, from traditional methods to the application of newer molecular methods such qPCR (Ward *et al*. 2004).

The development of disease progress curves from plant disease data is useful when disease incidence and/or severity can be measured over time from the onset of infection. They provide useful tools for understanding disease epidemics and can be used as models to predict disease risk (van Maanen and Xu 2003; Jeger 2004). Disease progress curves developed for plant diseases are usually based on visual scoring of either disease incidence or severity over time. However, with a disease such as botrytis bunch rot, the long latent phase of the pathogen means that waiting for visual symptoms limits control options, due to industry restrictions on fungicide timing applications. In addition, by this stage in fruit development the disease can

progress very rapidly under ideal conditions. The latent phase, therefore, is key to further understanding the disease.

Grape berry development consists of several key events in relation to pathogen development. These include flowering, berry development and berry ripening (Mullins *et al*. 1992). During flowering, *B*. *cinerea* can become established in the fruit by infecting the style, ovules, stigmas, stamens, petals or pedicel of the flower (McClellan and Hewitt 1973; Nair and Parker 1985, Keller *et al*. 2003; Elmer and Michailides 2004). Once *B*. *cinerea* has become established during the early stages of fruit development, it then goes into a latency phase during which there appears to be no active growth due to the presence of higher concentration of antifungal compounds that include stilbenes and phytoalexins (McClellan and Hewitt 1973; Verhoeff 1980; Keller *et al*. 2003; Pezet *et al*. 2003). During this latent phase between flowering and *véraison*, the host does not exhibit symptoms of infection. It is not until the berry starts to ripen that symptoms of botrytis bunch rot may start to appear in the infected tissue. Sugar accumulation (measured by total soluble solids) is associated with expression of botrytis bunch rot (BBR); others include increasing pH resulting from decreasing concentration of organic acids (e.g. tartaric and malic acid), changes in tannin and phenolic compound levels and decrease in some antifungal compounds, allowing the fungus to excrete enzymes resulting in the visible rot (Hale 1968; Mullins *et al*. 1992; Wolf *et al*. 1997; Breuil 1999; Gabler *et al*. 2003; Pezet *et al*. 2003). The main pathway for fruit to become infected later in the season is via wounds that may arise from mechanical damage, insects, infection from other pathogens, and splitting due to rain or tight bunches (Nair and Parker 1985; Nair *et al*. 1988; Bailey *et al*. 1997; Gabler *et al*. 2003; Keller *et al*. 2003). For significant disease expression and spread during ripening, the presence of moisture such as rain, dew or humidity is needed (Gubler *et al*. 1987; Nair *et al*. 1988; Vail *et al*. 1998). If weather conditions are dry during ripening, latent infections may not progress any further than the initial infection and disease severity is less (Zitter & Wilcox 2007a; Evans 2008).

Currently, qualitative techniques such as moist incubation of plant tissue are used to monitor latent infections of plant diseases such as BBR to gain estimated incidence of the disease in the crop (Holz *et al*. 2003; Cadle-Davidson 2008). These bioassays can

determine the presence or absence of the target organism, but cannot be used to accurately quantify the severity or degree of colonization. The moist incubation method involves sampling the host tissue and incubating the tissue for several days with moisture until the fungus grows and sporulates on the surface of the tissue. The method is simple and relatively low cost in setting up; however, it is time-consuming and relies on a trained operator to identify the plant pathogens using microscopy.

For the detection and monitoring of plant diseases, there has been a recent shift to using molecular-based methods due to their accuracy and rapid turn-around time as opposed to the traditional incubation methods (Ward *et al*. 2004). Real-time quantitative PCR (qPCR) is a molecular-based technique that has been used for diagnostic purposes, pathogen quantification, gene expression studies, and population diversity studies in plant pathology (refer to Chapters One and Two for a detailed explanation of the technique) (McCartney *et al*. 2003; Gachon *et al*. 2004; Schena *et al*. 2004; Valasek and Repa 2005). The main advantage of qPCR over the traditional incubation methods is that it can quantify the amount of colonisation of the target pathogen within the plant tissue at that particular sample point, without the need of the sample to show signs of infection (Gachon et al 2004; Gao *et al*. 2004; Schena *et al*. 2004; Hayden *et al*. 2006; Coolong *et al*. 2008; Minerdi *et al*. 2008). The method does not require a large sample volume, and the turnaround time can be quicker than that of the incubation methods (Gachon *et al*. 2004; Schena *et al*. 2004).

The qPCR technique has been used to detect *B*. *cinerea* in plant and fruit samples (Brouwer *et al*. 2003; Mehli *et al*. 2005; Suarez *et al*. 2005; Cadle-Davidson 2008; Celik *et al*. 2009; Diguta *et al*. 2010). Brouwer *et al*. (2003) tested a duplex SYBR based qPCR assay to study the temporal progression of several pathogens, which included the fungal pathogens *B*. *cinerea* and *Alternaria brassicicola* on the host *Arabidopsis thaliana*. The study showed that the technique could detect both pathogens on the host, from initial infection to the stages at which the plant was showing significant symptoms. It also discussed the effect that infected tissue would have on DNA quality, due to the nature of the pathogen being a necrotroph. Necrotrophic plant pathogens prefer to colonise dead or decaying tissue, they can release enzymes to speed up the decaying process within the plant cells. This process could have an adverse effect on the quality and yield of the DNA taken from the

infected plant tissue. The study highlighted the potential of molecular technology for tracking a plant pathogen from initial latent infection or identifying fungicide resistant strains.

Celik *et al*. (2009) developed a SYBR qPCR assay to quantify *B*. *cinerea* in symptomatic and asymptomatic table grape berries after harvest and during storage (refer to Chapter One, Section 1.9 for further information). Diguta *et al*. (2010) also developed a SYBR qPCR assay to quantify the number of spores on grape samples at a physiologically ripe stage that had been subjected to a fungicide control trial. The samples were washed to obtain the *B*. *cinerea* spores from which the DNA was extracted. The assay did not use whole berries to extract DNA and therefore did not account for internal colonisation by the fungus. Cadle-Davidson (2008) tested both a published assay by Rigotti *et al*. (2002) and developed another Taqman® (hydrolysis) probe assay to quantify the amount of *B*. *cinerea* in naturally infected grape berries for a number of different *Vitis* and hybrid species. The study also compared samples from several different vine growth stages, including pea size, bunch closure, *véraison* and harvest. However, the study did not use the qPCR assay to develop temporal progression curves; rather the assay was developed and used to test the potential of the assay to detect *B*. *cinerea* at the different growth stages when fruit are not showing signs of infection. Cadle-Davidson's study demonstrated the potential of the qPCR technique for studying temporal progression of *B*. *cinerea* in grape berries by the quantification of the target DNA, whereas the previously mentioned studies only applied the technique to samples at harvest.

The application of qPCR has the potential to provide a greater understanding of the temporal progression of infection by *B*. *cinerea* in grapes from the initial stages of infection until harvest. By using field material of two commercial varieties of wine grapes, there were two main objectives of this study: 1) to use the qPCR assay developed in Chapter Two to develop temporal progress curves of the amount of *B*. *cinerea* DNA and *V*. *vinifera* DNA from pre-bunch closure until harvest, and 2) to determine whether or not there was any correlation between the qPCR results and visual assessments of bunch rot during the period of berry ripening.

3.2. Methods

3.2.1. Field site and berry sampling

Grape berry samples were collected during the 2008-09 growing season from small plot trials with Riesling and Sauvignon Blanc situated in the Coal River Valley wine-growing region of Tasmania. The small plot trials were set-up in a randomised block pattern with each treatment replication (experimental unit) consisting of a panel of 5-7 vines, with 8 treatments and six replicate blocks. Trials were part of a fungicide timing experiment, which fed into a larger project focused on developing a model for predicting the risk of BBR (Evans *et al*. 2010b). The 'nil fungicide' treatment plots on each of the trial sites were used to obtain the samples. Sampling occurred at six key growth stages based on the modified Eichhorn and Lorenz (EL) system, which included pre bunch closure, *véraison*, ripening (3 stages) and harvest (Table 3.1) (Coombe 1995; Lorenz *et al*. 1995). A sample of fifty berries was randomly selected from each panel of vines per treatment replication. After sample collection at each time point, the berry samples were stored in a -20°C freezer until processing could take place (approximately 12 months later).

3.2.2. DNA extraction and duplex qPCR

All grape samples were processed according to the methods in Chapter Two. Prior to DNA extraction, the fifty berry samples were snap frozen with liquid nitrogen and ground to a fine powder using a mortar and pestle as described in section 2.2.1 (Field sample collection and processing). The DNA extraction method and cleaning procedure used was that previously described in sections 2.2.2.1 and 2.2.2.2. Samples were cleaned to remove possible PCR inhibitors present in samples. Where DNA concentration was above 5 ng/µL after quantification with PicoGreen® (Invitrogen Pty Ltd) (refer section 2.2.2.3 DNA Quantification), samples were normalised to this amount.

After standardisation, the amount of *B. cinerea* DNA was quantified using the duplex qPCR assay developed in Chapter Two (see section 2.2.5). Each sample was tested in duplicate with each reaction containing the following: - 12.5 µL 2X StrataGene

Brilliant® II qPCR Master Mix (StrataGene, Agilent Technologies, California, USA), 0.3 µM of each primer (KJD BcF, KJD BcR, KJD GF and KJD GR), 0.2 µM of each probe (KJD BcP and KJD GP), 2.5 µL of DNA sample, and sterile Milli Q water to a total volume of 25 µL. Samples were quantified using an optimised dilution series containing both *B. cinerea* DNA and *V. vinifera* DNA (refer section 2.2.5.5). A RotorGene 3000 real time machine (Corbett Life Sciences Pty Ltd) was used for the qPCR reactions as previously stated (section 2.2.5.3). For each qPCR run, the following controls were included for the *B. cinerea* primer set: four water (no template) negative controls, two *V. vinifera* DNA (5 ng/uL) negative controls and an undiluted *B. cinerea* DNA (5 ng/µL) as a positive control. For the *V. vinifera* primer set that formed part of the duplex assay, the *B. cinerea* DNA provided a negative control and *V. vinifera* a positive control. Three consecutive qPCR runs were completed for the analysis of the field samples. Samples were duplicated within each run.

Table 3.1: Dates at which sample collection occurred for the 50-berry samples along with the associated modified Eichhorn and Lorenz (EL) growth stage (Coombe 1995) of the vines and days before harvest for both *Vitis vinifera* cvs. Riesling and Sauvignon Blanc.

Sample Point	Sample Date	Riesling		Sauvignon Blanc	
		EL Stage	Days from harvest	EL Stage	Days from harvest
1	19/01/2009	Pre Bunch Closure (EL32)	92	-	-
	4/02/2009	-	-	Pre Bunch Closure (EL32)	55
2	26/01/2009	*Véraison* (EL35)	85	-	-
	26/02/2009	-	-	*Véraison* (EL35)	33
3	6/03/2009	Ripening (EL36)	46	Ripening (EL36)	25
4	9/03/2009	Ripening (EL37)	43	Ripening (EL37)	22
5	16/03/2009	Ripening (EL37)	36	Ripening (EL37)	15
6	31/03/2009	-	-	Harvest (EL38)	0
	21/04/2009	Harvest (EL38)	0	-	-

3.2.3. Visual assessments

Visual assessments of disease severity were performed during the growing season from *véraison* until harvest. The Riesling trial was assessed on six dates, while the Sauvignon Blanc trial was assessed on five dates. Disease severity of bunches was scored as the percentage of visibly infected berries in the bunch (Refer to Appendix D

for the figure of Bunch Scoring) (Refer to Table 3.3 for dates of assessments). A total of 30 bunches per replication (6 reps) were used to obtain mean severity scores. The same bunches were used for each of the assessment dates.

Table 3.2: Dates of visual assessments for both Riesling and Sauvignon Blanc commencing at the beginning of ripening, from the modified EL stages 35 to 38.

Assessment Point	Riesling	Sauvignon Blanc
1	09/03/2009	09/03/2009
2	16/03/2009	16/03/2009
3	24/03/2009	20/03/2009
4	02/04/2009	26/03/2009
5	16/04/2009	30/03/2009
6	20/04/2009	-

3.2.4. Data analysis

The qPCR data was collected using the RotorGene software supplied with the real-time PCR machine and analysis of raw data was performed using a later version of the software RotorGene Q, Pure Detection (version 1.7, Build 94) (Qiagen Pty Ltd). Mean cycle threshold (Ct) value was calculated and then used to calculate the amount of DNA as described in Cadle-Davidson (2008).

To derive the linear equation, the known amounts of standard for each reaction were log transformed. The equation used for the transformation of the known standards in the dilution series is shown in Equation B3 (Appendix B). A linear equation was determined from the standards in order to calculate the amount of *B. cinerea* DNA found in the field samples (refer to Equation B4, Appendix B) and then transformed back to actual amounts using equation B5 (Appendix B). All values were calculated using nanograms (ng) as the standard unit. The reaction efficiency was also calculated using the equation by Bustin *et al*. (2009) which is shown in equation B2 (Appendix B) using the calculated slope value from the linear equation derived from the standard dilution series.

Statistical analysis involving linear regressions, standard error (for $n \geq 3$), mean Ct and *B. cinerea* DNA amount were calculated for each sample for both standard dilution series and field samples. GenStat® 10th edition version 10.1 (VSN

International Ltd, UK) was used to conduct general linear regression analysis for each of the curves. General linear model regression analysis was also used to compare curves using the same software. All temporal graphs were generated using Microsoft Office Excel® and trend lines produced.

3.3. Results

3.3.1. DNA extraction and quantification

DNA was extracted in detectable amounts for 66% of the grape berry samples. The amount of DNA extracted varied between varieties over the different growth stages (Table 3.3). For Sauvignon Blanc the DNA yields at each sample point were similar (Table 3.3). In the Riesling samples, the DNA concentration at PBC was 2.32 ng/µL, and then it dropped to below detectable levels until harvest, when the mean concentration was 2.46 ng/µL. When DNA concentration was below the detection threshold (Riesling sample points 2 - 5), a nominal concentration of 1 ng/µL was used.

Table 3.3: Mean DNA concentration (prior to qPCR analysis) at Eichhorn and Lorenz (EL) stages for both Sauvignon Blanc and Riesling. Standard error (SE) is also shown. Refer to Table 3.4 for further detail about the qPCR analyses..

Sample Point (EL stage)	DNA amount for Sauvignon Blanc (ng/µL) (SE)	DNA amount for Riesling (ng/µL) (SE)
1) Pre Bunch Closure (EL 32)	2.97 (0.08)	2.32 (0.66)
2) *Véraison* (EL 35)	2.71 (0.04)	1 (0) [a]
3) Ripening (EL 37)	2.62 (0.12)	1 (0) [a]
4) Ripening (EL 37)	3.05 (0.21)	1 (0) [a]
5) Ripening (EL 37)	3.05 (0.03)	1 (0) [a]
6) Harvest (EL 38)	3.03 (0.07)	2.46 (0.31)

[a] Quantification method was unable to detect DNA for these samples.

3.3.2. Standard dilution series for qPCR

In all three qPCR runs, the standard dilution series for *B. cinerea* DNA consistently obtained high R^2 values (above 0.98). The reaction efficiencies for the three runs were 97%, 84% and 95% (Figure 3.1). Linear regression analysis of the standard

curves generated found that there was no significant difference between the runs (P >0.05). Where *V. vinifera* DNA was present in standards (excluding *B. cinerea* stock and water) Ct values were obtained. However a linear curve could not be generated for the *V. vinifera* standards (control) as the Ct values were too close.

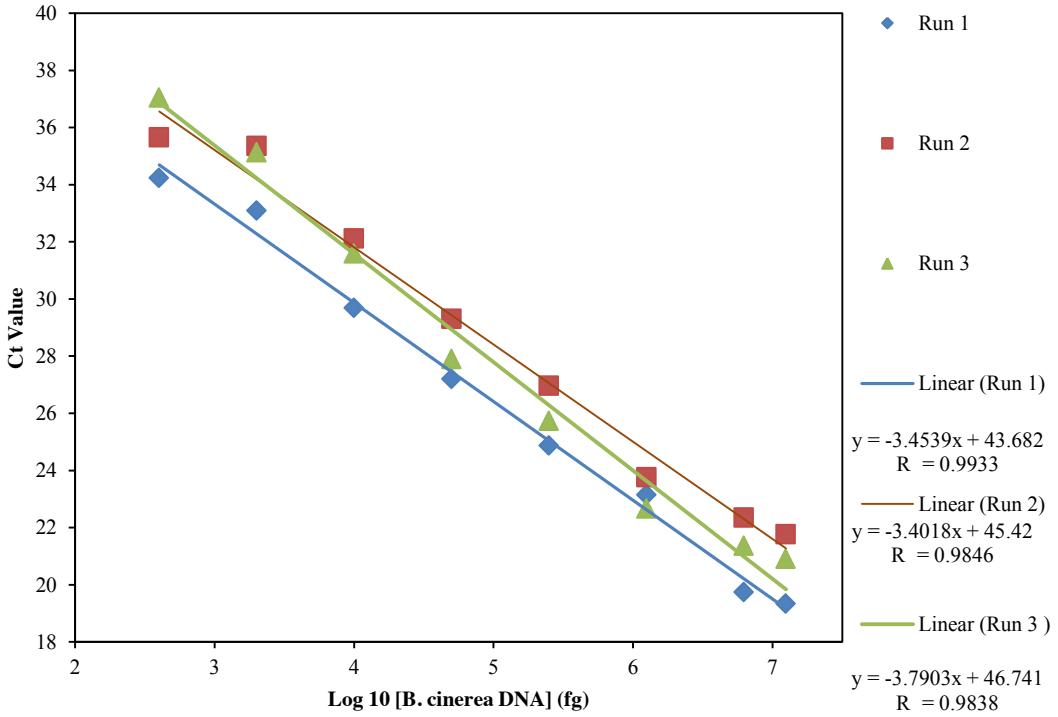

Figure 3.1: Linear regressions for the dilution series standards used to quantify the *Botrytis cinerea* DNA in the field samples for each qPCR run. Data points used for linear regression represent the mean Ct value for duplicate samples. Linear equations and R^2 values for each run are provided.

3.3.3. The detection and temporal progression of B. cinerea in grape berries

The qPCR assay was able to detect *B. cinerea* in both cultivars (Riesling and Sauvignon Blanc) at each of the growth stages from PBC to harvest, compared to visual assessment, which only detected *B. cinerea* during ripening. However, detection via the qPCR method was found to be inconsistent, as not all of the samples produced either positive or valid results. Of the 69 samples tested, five samples failed the qPCR test (7%), because the assay was unable to detect either *B. cinerea* DNA or the control (*V. vinifera* DNA) (Table 3.4). Two samples from the Riesling trial were found to be outliers as the results indicated a very high amount of *B. cinerea* DNA. This resulted in the samples being removed from the data set prior to temporal curve

analysis (Refer to Table 3.4 for summary). The lowest detectable concentration of *B. cinerea* DNA by qPCR was 50 fg in a sample from the Sauvignon Blanc site at ripening (Point 4, 9/03/09 (22 days from harvest). The highest detectable concentration of *B. cinerea* DNA by qPCR was 2.8 ng in a sample taken from the Riesling site at harvest (Point 6, 21/4/09 (0 days from Harvest)).

Table 3.4: Summary of total number of samples tested for both varieties, Sauvignon Blanc (SAB) and Riesling (RIE). The sample number for analysis includes those samples that were used to generate the temporal curves, after discarding replicates that had missing data due to failed qPCR (neither *Botrytis cinerea* or *Vitis vinifera* DNA was detected) or outliers with *Botrytis cinerea* DNA quantities well beyond the range of most samples.

Variety	Sample Point	No. of Plots	Total No. Samples qPCR	Post qPCR		
				Failed qPCR	Outliers Removed	Sample No. for Analysis
SAB	1	6	6	0	0	6
	2	6	6	1	0	5
	3	6	5[a]	0	0	5
	4	6	5[a]	0	0	5
	5	6	6	0	0	6
	6	6	6	0	0	6
	Total		34	1	0	33
RIE	1	6	6	0	1	5
	2	6	6	2	0	4
	3	6	6	0	0	6
	4	6	6	1	0	6
	5	6	5	0	1	4
	6	6	6	1	0	5
	Total		35	4	2	29
Grand Total			69	5	2	62

[a] **Samples were missing prior to DNA extraction or lost during the extraction process.**

Temporal progress curves were generated for $\log_{10}$ [*B. cinerea* DNA (fg)] and visual disease assessment scores for both Riesling and Sauvignon Blanc (Refer to Figures 3.2 and 3.3). In the Riesling, the amount of *B. cinerea* DNA appeared to remain relatively constant over time with no significant differences between sample points as reflected in the trend line (Figure 3.2). In contrast, the temporal progression curve developed for Sauvignon Blanc had fluctuated significantly in the amount of DNA between sample points two (26/02/2009) to five (6/03/2009) (Figure 3.3). Overall, the Riesling samples appeared to have more *B. cinerea* DNA in the samples compared to that of the Sauvignon Blanc samples. The temporal progression of the visual

symptoms for both varieties increased throughout the ripening process (Figures 3.2 and 3.3).

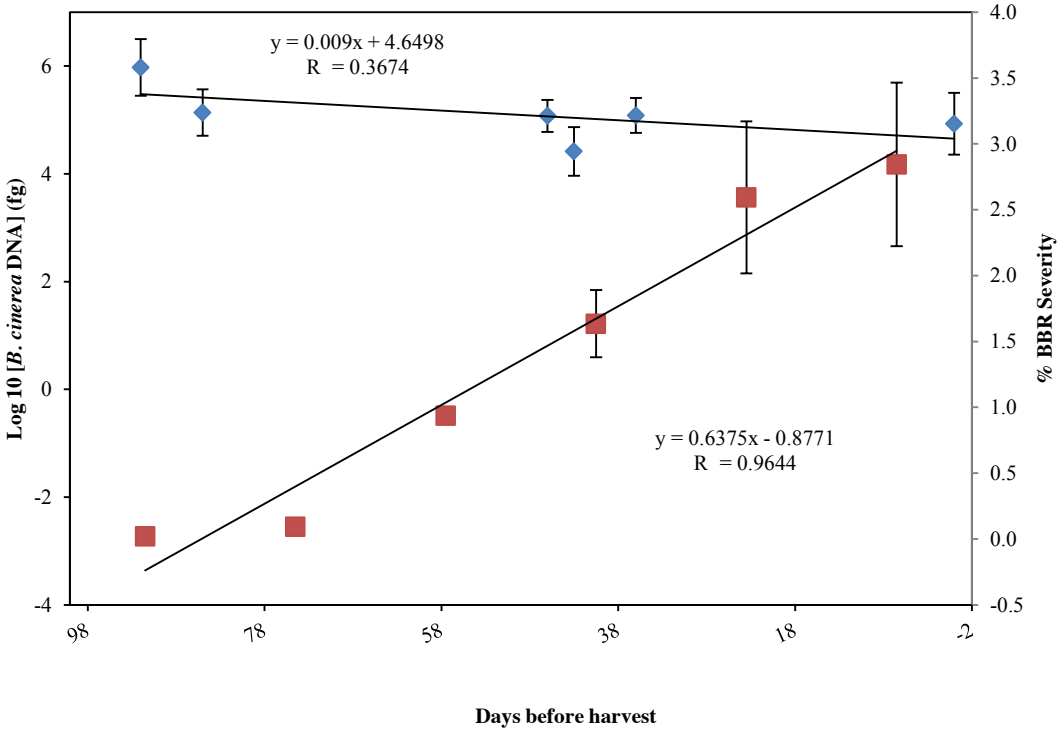

Figure 3.2: Temporal progression of Botrytis bunch rot development in Riesling during the 2008-9 growing season. The mean amount of *B. cinerea* DNA measured by qPCR (◆) and the mean percentage botrytis bunch rot (BBR) severity from ripening until harvest (■). Error bars represent standard error.

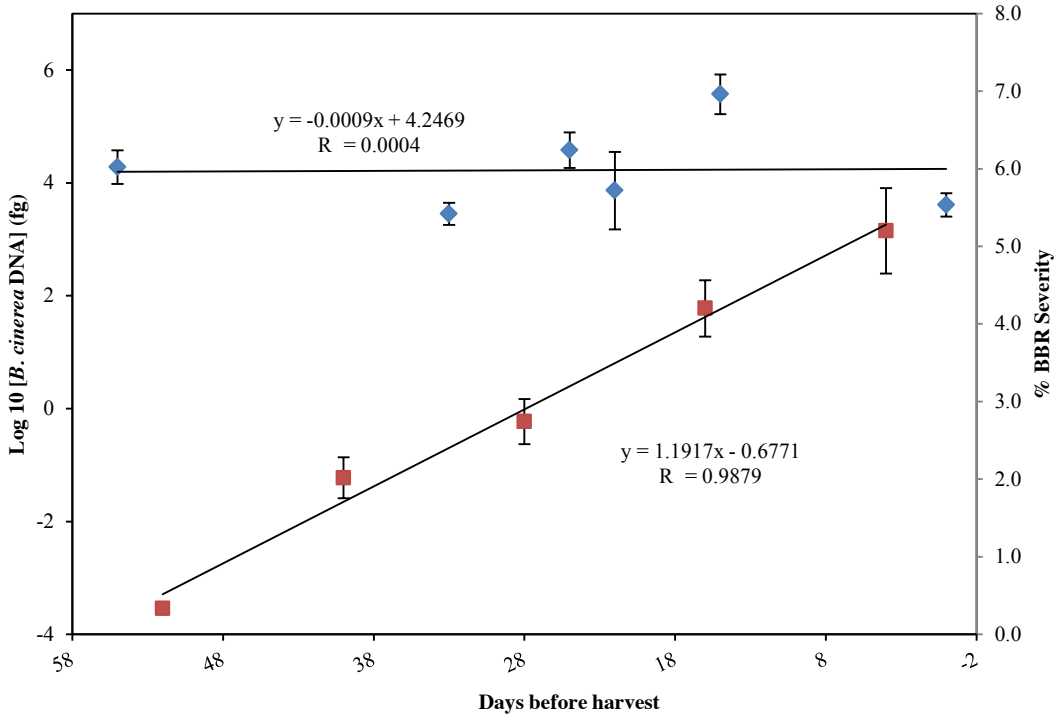

Figure 3.3: Temporal Progression of Botrytis bunch rot during the growing season using mean amount of *B. cinerea* DNA (◆) and visual assessments (%) in Sauvignon Blanc (■). To obtain the mean DNA concentration, were transformed to a log value. Standard error (SE) was calculated to show error bars.

3.3.4. Detection of V. vinifera DNA over time

The quantity and quality of DNA in a qPCR reaction both affect Ct value (Heid *et al*. 1996). Therefore, the Ct values were used to reflect the relative *V. vinifera* DNA quantity and quality for each of the sample points (Figure 3.4). There appeared to be variation in *V. vinifera* DNA across all sample points. The mean Ct values for the Sauvignon Blanc trial suggested that at Point 2 (Ct 26.41) samples had significantly greater DNA quantity and/or quality than the other time points (ranging from 32.85 to 36.36) (Figure 3.4). For Riesling, samples had significantly greater quantity and/or quality at Points 2 and 3 than the other time points. At points 4 and 6, DNA failed to amplify, suggesting that the *V. Vinifera* DNA quality was poor in the sample (Figure 3.4). This may have been due to PCR inhibitors being co-extracted during sample preparation.

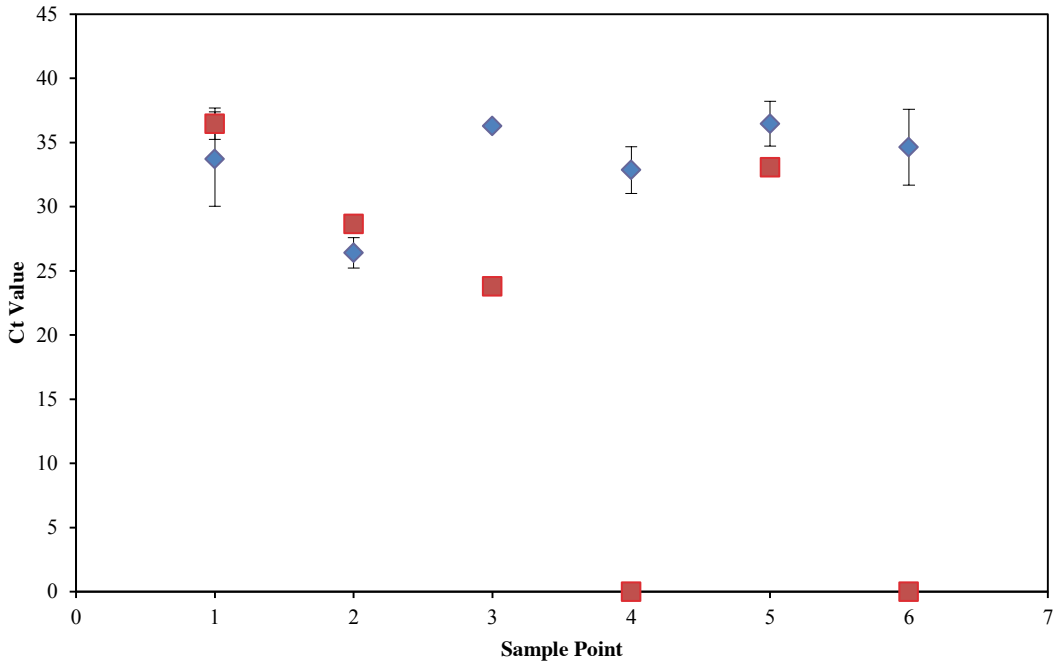

Figure 3.4: Mean Ct value for the detection of *Vitis vinifera* DNA (control) for each of the sample points for both Sauvignon Blanc (◆) and Riesling (■). Sample points include pre-bunch closure (1), *véraison* (2), ripening (3, 4, 5) and harvest (6). Higher Ct values (>35) and 0 represents lower amounts of *V. vinifera* DNA. Standard Error (SE) bars are also shown. Mean Ct values were calculated using the sample points where data was available from all six replicate plots.

3.4. Discussion

This study demonstrated the application of qPCR in studying the temporal progression of *B. cinerea* in grape berries. The qPCR assay successfully detected the fungus in naturally infected berries of *V. vinifera* cv. Riesling and Sauvignon Blanc, at key grapevine growth stages of PBC, *véraison*, ripening and harvest. The use of the qPCR technique was able to build on work published by Cadle-Davidson (2008), in which it was considered as a potential research tool to track fruit infection from establishment early in the season until harvest.

Like the study by Cadle-Davidson (2008), the qPCR assay used in this study successfully detected the fungus in grape berries throughout the season, from early stages of development until harvest. The sensitivity of the assay was shown since it was able to detect the latent infection of the fungus at PBC. It is at this crop stage that the only way of detecting or monitoring of the fungus is via moist incubation of the berries, which can be time consuming. In some cases, there may not be disease expression at PBC due to the presence of inhibitory compounds in the fruit that have not broken down (Cadle-Davidson, 2008). Unlike the qPCR results, the visual symptoms of the disease only became evident from ripening until harvest. During ripening, the fungus established in the berry is actively secreting the enzymes to break down tissue, while during early developmental stages of the fruit fungal growth is thought to be inhibited (Keller *et al*. 2003; Pezet *et al*. 2003).

The grape berry goes through chemical and physical changes during the growing season, which may have affected the yield and the quality of the extracted DNA. The growth and development of a grape berry is a complex process that involves both physical and chemical changes. Varma *et al*. (2007) noted that the chemical makeup of plant tissue has the ability to affect DNA yield and quality. Overall, the higher yields of DNA obtained at PBC would have been due to the berry going through active cell division, with secondary metabolites forming mainly from *véraison* onwards (Mullins *et al*. 1992; Varma *et al*. 2007). Molecules such as phenolics and sugars (carbohydrates) have been found to have a negative impact on DNA yield and quality and thus affect PCR results (Varma *et al*. 2007). The results from this study show that some samples taken after PBC produced quantifiable amounts of DNA,

which may have resulted from the berry transitioning from cell division to cell expansion where there is an increase in solute uptake (e.g. sugars). There is also the possibility that as *B*. *cinerea* becomes active within the berry, the excreting of fungal enzymes that break down the tissue would be oxidising the plant material leading to the degradation of the DNA within the sample, resulting in lower quality and yield of the DNA after extraction and cleaning steps (Varma *et al*. 2007). Further improvement of the extraction process might enable greater DNA yield and quality, and further reduce the risk of failed quantification and PCR results.

Variation in the amounts of total DNA per sample may have resulted from a number of aspects in the experiment design. One factor was sample size and the sampling strategy. Each berry was sampled randomly. Variation in the amount of *B*. *cinerea* DNA would be expected from this sampling strategy, as there was no guarantee that each berry sampled would have *B*. *cinerea* as this study looked at the natural infection of the fruit. Another possibility is that the DNA extraction method involved using a small sub-sample of the ground material, which may mean that the random sub-sample collected, may not have as much *B*. *cinerea* DNA, which may be present in the rest of the ground material. However, the fine powder produced was thoroughly mixed during the grinding process.

The temporal curves illustrated that there was no significant correlation between the amount of *B*. *cinerea* DNA and severity of visual symptoms. Relative to the visual symptoms, the total amount of *B*. *cinerea* DNA in the fifty-berry sample appeared to remain at similar levels throughout the season, despite the possibility that some variation in amounts of *B*. *cinerea* DNA could have been due to experimental design. This result suggests that infection without subsequent tissue colonisation is a characteristic of early latent infections of the fungus. The marked increase in symptoms during ripening may be due to enzymes that the fungus releases to break down the grape berry resulting in the pink brown rot (Bulit and Dubos 1988). Further investigations are warranted to improve understanding of the chemical changes that occur and whether or not the fungus is excreting enzymes to break down tissue without a corresponding increase in its biomass after latency. Moreover, future investigations need to consider the physical and biochemical changes that occur during grape berry development. Knowledge of the relative rates of plant host and

fungal growth at different stages of berry development might also provide an explanation for the flat response in the amounts of *B*. *cinerea* DNA observed.

The qPCR results for *V*. *vinifera* DNA measured at each sample point appeared to vary over time. The least amount of DNA as reflected in the Ct values of zero (no *V*. *vinifera* DNA detected) in the Riesling observed during the ripening phase corresponds to the time of cell enlargement when there is an increase in the uptake of sugars, which can become PCR inhibitors if co-extracted (Varma *et al*. 2007). The phases of berry development can be described as reflecting a double sigmoid curve (Coombe 1996, Harries *et al*. 1968, Coombe & Hale 1973). It can be broken up into three distinct stages of berry development (cell division), a lag phase of no active development/ berry growth and finally berry ripening, in which cell expansion occurs due to the uptake of sugars and other solutes (Coombe 1960; Coombe and Hale 1973; Winkler *et al*. 1974; Mullins *et al*. 1992; Symons *et al*. 2006). The lowest Ct values were observed during the period between berry development and the end of the lag phase, when the berry is not actively taking up solutes. In contrast, towards the later sample points Ct values were either higher or not recorded (on two occasions with Riesling). Lack of a DNA product indicated that either there was no or very little *V*. *vinifera* DNA extracted, or actually that there may have been inhibition occurring resulting in the assay's limited ability to detect the target DNA. As previously noted the sugars and other solutes readily stored by the berry can act as inhibitors in PCR reactions if co-extracted (Varma *et al*. 2007).

There are a number of factors that may have contributed to the qPCR assay failing to detect *V*. *vinifera* DNA for sample points 4 and 6 in the Riesling. One factor is that during the DNA extraction methods, DNA can be removed and there are potential risks that during the cleaning steps some DNA will be removed due to being bound to contaminants, which are targeted during this phase. Berries within a bunch, vine, or block of a single variety do not ripen equally over time, i.e. there is variation in sugar and acid levels. Thus, the grapes contain varying amounts of the complex sugars and carbohydrates, which for DNA extraction will affect the amount of impurities that will be co-extracted during the process and affect the end DNA yield after the use of cleaning kits. Furthermore, infection levels will vary between berries, which would also affect the amount of breakdown of berry tissue by *B*. *cinerea*. These factors

might affect DNA quality, which may have been the cause of the lower amount *V. vinifera* DNA detected at these points, as the samples were taken when the fungus was most likely to be the most active during the ripening period. Even though the assay did not always detect the *V. vinifera* DNA in the sample, the assay detected *B. cinerea* DNA, suggesting the DNA obtained from the extraction process was not affected by the degradation of the plant tissue by the fungus. This would enable a positive qPCR result to be reached even though the sample may contain *V. vinifera* DNA either at extremely low levels or that the quality, too poor to allow a reaction to occur to detect the *V. vinifera* DNA. Further investigation over a number of seasons using the qPCR to track the amount of grape DNA would help to provide insight to berry growth on a molecular scale. Moreover, there is potential to use the technique to improve understanding of the relationship between the berry and the invading *B. cinerea*, as well as for quantification the amount of fruit degradation.

The amount of DNA isolated was found to vary during the season, which highlights the complexity of the relationship between fruit development and *B. cinerea* infection.

3.5. Conclusion

Quantitative PCR was successfully applied to grape berry samples resulting in temporal curves being produced for *B. cinerea* DNA in two *V. vinifera* cultivars (Riesling and Sauvignon Blanc). *Botrytis cinerea* DNA was detected at all sample points from PBC up until harvest. The total amount of DNA extracted varied among sample points, due to the nature of the host tissue, with berry size increasing as the season progressed. The results highlight the complexity of the relationship between fruit development and *B. cinerea* infection and the effect on DNA levels (total, *B. cinerea* and *V. vinifera* DNA). There did not appear to be a significant correlation between BBR symptoms and *B. cinerea* DNA mass. Further investigation is needed with a direct comparisons between visual scoring and the quantification via qPCR to fully understand the complex relationship between the two methods. Results suggest that throughout the season, fungal mass in terms of DNA does not vary greatly, with only a minor increase as fruit ripens, after initial infection during the 'latent period' between flowering and early berry development. Further investigation is warranted in

order to determine the extent of colonisation by the fungus during the early berry development phase and how that variability is correlated to BBR severity at harvest.

Chapter Four
Botrytis bunch rot epidemics & a comparison between novel indicators of *B. cinerea* infection in grape juice samples

4.1 Introduction

Botrytis bunch rot (BBR), caused by *Botrytis cinerea*, can result in major quality implications for the winemaker and grower. There are a number of aspects, which can ultimately affect the amount of BBR that is expressed at harvest. These include the various infection pathways of *B. cinerea* (Elmer and Michailides 2004), sources of inoculum (Jaspers *et al*. 2013), amount of inoculum, fungicide timing (Agnew *et al*. 2004; Edwards *et al*. 2009) and weather (Thomas *et al*. 1988). For detection and quantification of BBR, the most widely used method is field observation close to harvest date. Earlier in the season, latent infection can be determined via moist incubation in laboratory tests (Holz *et al*. 2003; Cadle Davidson 2008). However, both methods can be time consuming, laborious, and requires symptoms and/or the pathogen to be visible. The methods also rely on the assessor's ability to distinguish between the different bunch rots that may be present and to subjectively assess the area of a bunch affected by bunch rot symptoms.

It would be of benefit to both industry and researchers to develop and test novel and accurate quantitative methods that correlate with and replace visual assessment of BBR (refer to Chapter One for detailed description of detection methods). There has been the development of field-based, portable Enzyme-Linked Immunosorbent Assays (ELISAs), which are still mainly used as a research tool for measuring BBR (Dewey *et al*. 2000; Obanor *et al*. 2002; Obanor *et al*. 2004; Dewey and Yohalem 2007; Dewey *et al*. 2008; Scott *et al*. 2010; Dewey *et al*. 2013). Independently, real-time quantitative polymerase chain reaction (qPCR) techniques have been developed

to measure *B*. *cinerea* in grape berries (Cadle-Davidson 2008; Celik *et al*. 2009; Scott *et al*. 2010) and spore surface load (Diguta *et al*. 2010, Scott *et al*. 2010). Recently there have also been investigations into the use of spectroscopy using the mid infrared wavelength (MIR) as a tool to measure BBR (Cozzolino *et al*. 2003; Versari *et al*. 2008; Scott *et al*. 2010). To date, there have been a limited number of published articles directly comparing qPCR and ELISA for the detection and monitoring of *B*. *cinerea* infection in fruit (Mehli *et al*. 2005; Celik et al. 2009). However, to date there has not been a similar comparison study in grapes, as studies have only investigated each of the methods independently.

Assessment of pest and disease damage on fruit by contracting wineries will often occur up to a week before harvest. This involves assessing the fruit while still on the vine, selecting at random a limited number of vines within the block and scoring damage in the fruit. However, sample size may result in the assessments not accurately representing the actual amount of disease present in grapes or reflect the effect that it will have on end wine quality. Depending on the severity of infection, *B*. *cinerea* can cause oxidation and taints in juice and wine (Peynaud 1984; Godden 2000; Godden 2003; Dumeau *et al*. 2004; Lorrain *et al*. 2012). Currently when fruit arrives at the winery, matter other than grapes (MOG) is assessed and the amount of highly infected fruit is recorded, which may result in separation of fruit load from others that are in the same quality band. The juice parameters that are assessed are total soluble solids (°Brix), pH and titratable acidity (TA), as well as colour (anthocyanin content) for red wine varieties (Krstic *et al*. 2003). To date, the qPCR assays that have been developed for quantification of *B*. *cinerea* in grapes have only been used on grape berries and not grape juice or must (crushed grapes) (Cadle-Davidson 2008; Celik *et al*. 2009; Diguta *et al*. 2010). However qPCR has successfully been used to detect powdery mildew (*Erysiphe necator*) in both grape must and juice (Stummer *et al*. 2006) and spoilage organisms (bacteria and yeasts) found in wine (Gindreau *et al*. 2001; Delaherche *et al*. 2004; Culbert *et al*. 2008). Stummer *et al*. (2006) was able to accurately detect 50 pg of *E*. *necator* per 100 ng of grape sample. The study also found it was only able to detect the fungus in unclarified juice and must, but not clarified juice or wine. These studies highlight the potential use of qPCR to detect and quantify *B*. *cinerea* in grape must or unclarified juice samples.

ELISA-based methods to detect *B*. *cinerea* in grape must/ juice have been developed as commercial kits in the last decade. ELISAs have shown to be a quick tool to detect *B*. *cinerea* in juice, with the main application being at the weighbridge to determine the amount of *B*. *cinerea* in the juice (Ricker *et al*. 1991; Dewey *et al*. 2000; Dewey *et al*. 2005; Envirologix 2007; Dewey *et al*. 2008). However they only provide a semi-quantitative measurement, and only work well when the fungus is actively growing within the fruit (Ward *et al*. 2004; Boonham *et al*. 2008; Celik *et al*. 2009). The ELISA works on the basis of recognising enzymes released by the invading fungus and therefore requires active fungal growth secreting enzymes to break down the host tissue.

Recent developments in spectroscopy methods, to rapidly assess grape, juice and wine quality, have highlighted its application in determining wine and juice quality parameters (*et al*. 2003; Cozzolino *et al*. 2007b; Versari *et al*. 2008; Cozzolino *et al*. 2010; Gishen *et al*. 2010; Scott *et al*. 2010). The benefits of these methods are that they are quick, and sample preparation is simple and potentially non-destructive (Gishen *et al*. 2005; Cozzolino *et al*. 2007a; Cozzolino *et al*. 2007b; Cozzolino *et al*. 2007c). Spectroscopy involves measuring the absorbance / reflectance of wavelengths from a sample in the UV, visible, near infrared (NIR) or mid-infrared regions (MIR). Samples can be in either a gaseous, liquid or solid form. Currently there are methods under development and in use for simultaneously determining pH, total soluble solids, anthocyanin content and monitoring of bottled wine during storage (Kennedy 2002; Cozzolino *et al*. 2003; Dambergs *et al*. 2007; Gishen *et al*. 2010; Ugliano *et al*. 2010). The potential to use spectroscopy for assessment of BBR has been investigated in Australia (Cozzolino *et al*. 2003). However these investigations in Australia have been limited, and are yet to result in standardised methods for measuring the levels of BBR in grapes for both research and commercial purposes. In this study, the MIR wavelength was chosen as it does not penetrate into the sample as far as NIR resulting in potentially less error, than that of NIR. In comparison when using NIR, there is a risk of greater error for samples that are not clarified as the NIR also gives overtones of the MIR wavelength. The MIR wavelength has also been successfully used in a study looking at fruit quality in relation to BBR at harvest as it is associated with gluconic acid (Versari *et al*. 2008).

Interpretation of the spectral readings of samples requires the use of multivariate analysis, which is commonly referred to as chemometrics (Cozzolino *et al*. 2007). The analysis enables the consideration of different variables simultaneously (Batten 1998; Cozzolino *et al*. 2007). There are several data analysis techniques that can be employed, with principal component analysis (PCA) being the most common. PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated latent variables called principal components (PCs). The data are reduced to a new set of values that best describe the difference between the samples. The first PC accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. In a plot of PC scores, clustering of related samples can be observed and relationships between variables can also be observed. When overlaid with sample information, the score plots can identify possible variation in the data related to experimental treatments.

Botrytis cinerea is a cosmopolitan necrotrophic plant pathogen that has the ability to infect at multiple stages during the season using a variety of infection pathways. The two main infection pathways in grapes are the 'early season' latent infection pathway and the 'mid to late season' necrotic tissue pathway (Elmer and Michailides 2004). Prior research has shown that each region and vineyard varies as to the predominant infection pathways and sources of inoculum (Wolf *et al*. 1997; Elmer and Michailides 2004; Jaspers *et al*. 2013). Understanding the key sources of inoculum within a vineyard is important in order to implement strategic control measures for *B*. *cinerea*. Investigations into the role of canopy trash as an inoculum source have found that although it may play an important role (Wolf *et al*. 1997; Rozario *et al*. 2005; Jaspers *et al*. 2013), the severity and incidence of BBR at harvest is highly weather dependent, requiring cool weather with free moisture or high relative humidity (Gubler *et al*. 1987; Nair *et al*. 1988; Thomas *et al*. 1988; Nicholas *et al*. 1994; Vail *et al*. 1998). As BBR severity increases over time, understanding it's temporal progression may help one to understand the developments of epidemics. The increase of any plant disease is not uniform and there would be environmental and control factors that will influence its progression over time.

Fungicide efficacy for controlling any plant disease is determined by a number of factors. As well as crop factors, weather, fungicide output rates (dilution factor (ratio of pesticide to water), application rate) and timing of application all determine how well a fungicide will perform. Over the last ten years, BBR research has focused on a more targeted approach to fungicide application and timing (Mundy and Beresford 2002; Agnew *et al*. 2004; Edwards *et al*. 2009). These studies have confirmed that there is a regional variation in the optimal time / growth stage at which to apply fungicides. They also showed that using a strategic plan based on the understanding of weather patterns, cultural practices and optimisation of fungicide timing can provide for effective control with reduced inputs compared with traditional programs of spraying at each allowable growth stage (flowering, PBC, *véraison*) (Agnew *et al*. 2004).

There were several aims of a field trial conducted at a single vineyard site and season:-

The first aim was to investigate the main infection pathway for *B*. *cinerea* through implementation of a fungicide timing trial. Two main infection pathways were defined as (a) the 'early season' latent infection pathway, where floral parts become infected during flowering up to full bloom then remain quiescent until fungal growth is activated during *véraison*, and (b) the 'mid to late season' necrotic tissue pathway where the inoculum sources are colonised necrotic plant tissue in the canopy (e.g. floral and bunch trash, sporulating rotten berries) and infection occurs directly or via wounds in developing berries. The role of sporulation in the spread of BBR was also investigated in the investigation of the role of fungicide timing. The second aim was to detect *B*. *cinerea* or indicators of BBR in grape juice/must using qPCR for sensitive quantification of *B*. *cinerea*, an ELISA-based QuickStix™ for semi-quantitative purposes and spectroscopic methods. The third aim was to determine if there was any correlation between the detection methods and BBR severity and incidence and juice characteristics (pH, titratable acidity, total soluble solids).

4.2. Methods

4.2.1. Trial Set-up

The field trial was established in a vineyard block consisting of *V. vinifera* cv. Chardonnay (G9V7) that was planted in 1998; the fruit was destined for the premium table wine market (Figure 4.1). The trellis system was Scott Henry, which consists of one arm on one wire with shoots trained upwards, while the second arm is on a lower wire and the shoots trained downwards. The field trial was composed of eight treatments (Table 4.1) with six replicate plots, which are described below. Each plot consisted of five vines. The vine and row spacings were 2.4 and 1.35 m, respectively. The design of the trial site was a randomised block layout, generated using the statistical software package 10th Edition (VSN International Ltd, UK). The six blocks were located in six adjacent rows.

The fungicide Switch® (Syngenta Group, Basel, Switzerland) containing the active ingredients of 375 g/kg cyprodinil plus 250 g/kg fludioxinil was used in the mid-season spray treatments of pea size (EL30-31) and PBC (EL32-33) (treatments 2-6), and was chosen due previous studies by Zitter & Wilcox (2007b) and Evans *et al*. 2010a) where it appeared to be effective in eradicating latent infections of *B. cinerea*. The fungicides were applied on the 2nd and the 22nd of January 2008 respectively. The second fungicide selected was trifloxystrobin (Flint®; Bayer Crop Science), however this particular fungicide is not registered for use in controlling BBR (Table 4.1). The fungicide was selected because of an anecdotal report that it might be effective in suppressing sporulation by *B. cinerea* (Wayne Wilcox, Cornell University, personal communication). This fungicide treatment was an addition to the trial investigating the role of spray timing has in minimising BBR. The fungicide was applied on the 18th February and the 13th March 2008. The fungicides were applied with a hand-held spray gun connected to a hose reel and diaphragm pump mounted on an all-terrain vehicle (ride –on quad bike). Application rates of the fungicide are given in Table 4.1. A non-ionic surfactant was used when the cyprodinil + fludioxinil fungicide was applied as it is recommended on the label when applying this particular fungicide to maximise spread and retention. This trial investigated the role of spray

time as such, only the treatments applying cyprodinil + fludioxinil at the two difference time points are directly comparable.

Standard commercial vineyard management practices were applied to the block, except that the spray program was modified so that no botryicides were sprayed on the trial section or the two buffer rows / panels on either side of the trial site. The vines were leaf plucked on the 20th December 2007 at the phenological stage of berry set (modified EL stage 27-29) (refer to Coombe (1995) for grapevine growth stage explanation). Immediately prior to *véraison*, the vines were hedged before installation of bird netting.

Canopy trash consisting of dead flower organs (calyptras, aborted berries) was removed from selected treatments using a compressed air blower set at 500 kPa, aimed at the bunch zone. Trash removal was performed twice during the growing season: on 12th December 2007 (EL stage 26-27- capfall complete) and 15th January 2008 (EL stage 27-31- berry set- pea size) (Table 4.1). The trash was collected from treatments 1 and 7 (Table 4.1), placed in plastic bags and taken to the laboratory. Trash was collect using plastic film placed under vine and then carefully transferred into bags to limit loss. A quantity of 100 calyptras and 100 aborted berries per replicate were placed on moist sterile filter paper in new Petri dishes spread across five plates per tissue type. The plates were incubated at room temperature under 12 h light / 12 h dark and the trash assessed for sporulating *B. cinerea* after 6 days.

Table 4. 1: Outline of treatments for the Small Plot Trial (2007-2008) showing the fungicide type and timing, and canopy trash removal. CF = capfall, PBC = pre-bunch closure. The dates of fungicide application for each treatment is shown under the respective growth stage. Application rates and total volume used is shown. The fungicide Switch® requires an adjuvant (Activator®) to ensure optimal retention by plant tissue after application.

			Fungicide Application					
Trt	Growth stage	Trash removal at 100% CF & prior to PBC	Pea size (EL 30-31) 2nd Jan, 2008	PBC (EL 32-33) 22nd Jan, 2008	*Véraison* (EL 34- 35) 18th Feb, 2008	Pre-Harvest (EL 36) 13th Mar, 2008	Rates	Total Volume Used
1	Nil	Yes	-	-	-	-	-	-
2	Nil	No	-	-	-	-	-	-
3	Pea-size	Yes	Switch® Activator®	-	-	-	80 g/100 L 20 mL/100 L	40 L
4	Pea-size	No	Switch® Activator®	-	-	-	80 g/100 L 20 mL/100 L	30 L
5	PBC	Yes	-	Switch® Activator®	-	-	80 g/100 L 20 mL/100 L	30 L
6	PBC	No	-	Switch® Activator®	-	-	80 g/100 L 20 mL/100 L	30 L
7	Late	Yes	-	-	Flint®	Flint®	16 g/100 L	-
8	Late	No	-	-	Flint®	Flint®	16 g/100 L	-

4.2.2.1. Environmental data collection

A weather station was placed on the eastern side of the block to collect environmental data for the growing season. Tinytag data loggers (Gemini Data Loggers Ltd, UK) were used to record mean air temperature, relative humidity, leaf wetness duration (surface moisture) and rainfall at 10 min intervals. The data loggers were located 1.6 m above the ground as described by Beresford and Spink (1992). The temperature and relative humidity data logger Tinytag Ultra 2 (dual channel) was housed in a plastic shelter (Hastings Data Loggers, HDL, Port Macquarie, Australia). A tipping bucket rain gauge (Rain Collector II, Davis Instruments, USA) adjusted to tip after every 0.2 mm of rainfall was used to collect rainfall data. The leaf wetness sensor (Model 237, Campbell Scientific Inc. Utah, USA) was mounted at a 10° angle in order to minimise surface moisture run-off and the cable modified to connect to a Tinytag data logger.

4.2.2.2. Visual assessments

Weekly visual disease assessments were carried out between 5th March and 7th April 2008. A total of 28 bunches (14 basal and 14 distal) were assessed and were tagged to distinguish them from sampling bunches. Basal bunches are bunches which are situated at position one on the shoot (above the shoot base), while distal bunches are secondary bunches on a shoot (adjacent to basal bunches). Bunches were assessed for BBR severity according to several categories: 1) pink brown plump berries, 2) shrivelled pink-brown berries 3) total BBR and 4) visible sporulation. The severity of other rots was also noted, as well as physical damage including sunburn. Bunches were scored on a percentage severity scale based on a key developed by Dr Bob Emmett (Department of Primary Industries, Victoria), which is in the Botrytis Check list (Cole *et al*. 2004) (refer to Appendix D). Incidence of BBR and other rots were also recorded; by observing the number of bunches which had the presence of disease using the same tagged bunches.

4.2.3. Sample collection during the season

4.2.3.1. Over Night Freezing and Incubation Technique (ONFIT)

The ONFIT technique was used to determine the amount of latent *B. cinerea* infections in the berries, as described by Evans *et al*. (2010b), which was a modification of the method developed by Lou and Michailides (2001). Eight bunches (four basal and four distal) per treatment replicate were sampled on 14th January 2008, just prior to the application of the PBC spray for treatments 1-4 only. Bunches were placed in labelled bags as per treatment, replication and bunch position. The aim of the sample was to determine if the application at pea-size had an effect on latent infection incidence in comparison with the untreated plots. A second sampling occurred at *véraison* for all treatments (1-8) on the 19th February 2008. At this time, only six bunches (three basal and three distal) were sampled per treatment replication due to low bunch numbers at the trial site.

The setup procedure for ONFIT is as follows: - bunches were taken back to the laboratory and placed in a – 20°C freezer for at least 24 h to breakdown the fungal inhibitors present in the berry tissue. After the freezing step, the bunches in each replicate bag were surface-sterilised using 70% ethanol for 10 sec followed by immersion in 1% sodium hypochlorite solution for 1 min. Twenty whole berries with pedicels attached were randomly cut using surface-sterilised scissors and forceps (sterilisation using 70% ethanol). Each 20 berry sample were placed on moist paper towels and held in position using a piece of rubber mesh ("rug hold" underlay) inside clean plastic containers (17.5 × 12 × 4 cm, approximately 500 mL) and sealed with a lid. The trays were incubated on the laboratory bench at room temperature (15 - 20°C) with exposure to natural daylight from windows. A total of 8 containers (4 basal bunches and 4 distal bunches) per treatment replication for treatments 1-4 were used for the PBC growth stage. This number of containers was reduce to 6 per treatment for the *véraison* assessment where bunches from all 8 treatments were examined. Containers were placed on the bench in either stacks of 4 and 3 due to limited bench space. Assessments were conducted after 9 and 13 days of incubation for the PBC sample point and 7 and 10 days for the *véraison* sample point. A stereomicroscope was used to help in the identification of *B. cinerea* and other

pathogens where possible. The incidence of berries with *B. cinerea*, *Aspergillus* spp., *Penicillium* spp., unidentified infection and no visible infection was noted.

4.2.3.2. Harvest samples

At harvest, six bunches (three basal and three distal) were randomly selected from the tagged visual assessment bunches for each replicate. Each bunch was bagged separately with its tag for identification and taken back to the laboratory. Bunches were incubated in their bags at room temperature for five days prior to assessment for *B. cinerea* sporulation, BBR severity and other diseases. Bunches were subjected to the natural light in the laboratory.

4.2.4. Bunch characteristics

Total soluble solids (TSS or °Brix) is used as tool to determine grape ripeness. During ripening from *véraison* onwards, sampling occurred weekly until harvest. A total of six replicate samples were taken at each time point. Each replicate sample consisted of a total of 32 berries, 4 berries per vine from a total of 8 vines. The vines which were used were the end vines of each of the treatments where there were no specifically tagged bunches that were to be used as part of the trial. The berries were placed inside a zip-lock plastic bag and squeezed manually to extract the juice. One mL of each juice sample was placed on the well of a digital pocket refractometer (Pocket PAL-1, Atago, Japan), which measured TSS.

Bunch compactness has been shown to correlate with BBR severity, with tight bunches often becoming severely infected (Hed *et al*. 2009). This was measured in treatment 8 only, six bunches (three basal and three distal) per replicate were harvested on 18th February 2008 at *véraison* (EL 35). Only treatment 8 was used, due to the limited number of bunches available and it was a preliminary study to determine if there was a relationship between the measure of compactness and BBR severity. The method used was that of Shavrukov *et al*. (2003). The wings of bunches were removed if they were well formed and not confused with the main rachis. The length and width of the bunch was measured using a set of digital

callipers. To determine volume, bunches were placed into a 250 mL tube filled with water and the volume of displaced water was measured. Using the equations from Shavrukov *et al*. (2003) the percentage bunch compactness (i.e. tightness) was calculated on a scale where 0% is a very tight compacted bunch (no free space) and 100% relates to no berries on a rachis (Equations B8.1 and B8.2, Appendix B). The visual assessment scores taken prior to harvest were then used to determine if there was a correlation between compactness and BBR severity.

4.2.5. Grape juice collection

Two samples (basal and distal), each containing six bunches were harvested from each treatment replication plot and stored in a cool box until transferred to a cool room (4 °C) until processed. The tagged bunches for the visual scoring of BBR severity and incidence were used at harvest to collect juice parameters. Tags were kept with harvested bunches in order to determine respective severity for each juice sample. Grape juice/must from each of the bunch samples was extracted using a 14 × 14 cm bench top aluminium stainless steel fruit basket press (Ferrari group, Italy) over 2 days. A 50 mL volume of juice was taken and sodium metabisulphite (200 mg/L SO_2) added to prevent oxidation for qPCR analysis at a later date. The sample was stored at –20 °C until qPCR analysis (section 4.2.5.3). Two × 2 mL juice samples were also retained and stored at -20°C for the immunological assay and MIR analyses (sections 4.2.5.2 and 4.2.5.4).

4.2.5.1. Immunoassay testing

An ELISA kit (QuickStix™, Envirologix Inc. Portland, Maine USA) was used to determine the amount of *B. cinerea* in grape juice, and the method followed the manufacturer's instructions. The assay used the monoclonal antibody Bc-12-CA4 to recognise the corresponding antigen specific to *B. cinerea* present in the juice/must (Dewey *et al*. 2000). Prior to testing juice samples taken at harvest, which were kept at -20°C until June 2008, were left on the bench to thaw until they reached room temperature. Samples were then prepared by homogenising 1 mL from each of the two 2 mL microfuge tubes of frozen juice per treatment replication.. Test kits were kept in the fridge at 4°C until required. The buffer solution and test strips provided in

the kits were removed from fridge left on the laboratory bench in order to reach room temperature. The juice sample was then mixed with the supplied EB8 buffer at a ratio of 1:5 after which a 500 µL aliquot was taken and placed in the provided test tube and a testing strip was placed in the solution. After 10 min of incubation at room temperature, strips were placed in the supplied reader and the signal intensity (SI) measured. SI is proportional to the amount of *B. cinerea* present in the juice samples, using a pre-programmed standard curve (the Dewey I-W Standard), which calculates *Botrytis cinerea* content in grape must on the basis of disease incidence in berries or via weight (Envirologix 2007; Dewey *et al*. 2008). The weight value of the amount of *B. cinerea* berries is calculated via multiplying the incidence level calculated from the reader by 0.333, where a half turgid *B. cinerea* infected berry weighs on average ⅓ of that of a healthy berry (Envirologix 2007; Dewey *et al*. 2008). The standards were derived from an initial grape juice stock consisting of 20 turgid infected berries and 80 healthy berries (Envirologix 2007). Juice samples were tested in triplicate.

4.2.5.2. DNA extraction and qPCR analysis of grape must

The DNA extraction method was modified for the extraction of DNA from grape juice samples, based on the methods reported by Lin & Walker (1997) and Cadle-Davidson (2008) (refer to Chapter Two, section 2.2.2). The DNA extraction buffers, Buffer A, Buffer B and Buffer C, were previously mentioned in Chapter Two and listed in Appendix A. All centrifugation steps were conducted with a Sorvall Super T21 SL5OR centrifuge (Thermo Fisher Scientific (formerly Kendro), Waltham MA USA) with a time period of 20 mins.

Fifteen mL aliquots of each juice sample were centrifuged at 3288 RCF to pellet the solids and the clear liquid decanted, after which samples were placed back in the –80°C freezer. Prior to DNA extraction, samples were then allowed to defrost slightly at room temperature for approximately 10 mins and 5 × 3 mm sterile stainless steel beads were added to each tube along with 10 mL modified Buffer A containing 3 % w/v PVP40 and 0.1 % v/v of beta-mercaptoethanol. Samples were then homogenised using a vortex, followed by centrifugation step at 3, 800 RCF at 4 °C.

The supernatant was discarded and the pellet re-suspended in a solution containing 2 mL each of Buffer A containing 0.1 % beta-mercaptoethanol), Buffer B and Buffer C. Samples were mixed using the vortex for 1 min and incubated in a water bath at 65°C for 30 min, being shaken by hand at 10 min intervals. Samples were then left to cool at room temperature for 5 - 10 mins prior to addition of 2 mL of 24:1 chloroform: isoamyl alcohol solution. Samples were shaken at 2 x 5 min intervals, and then centrifuged again (3, 800 RCF, temperature set at 4 °C). The top layers were collected and transferred into new Falcon tubes and another 2 mL of the chloroform: isoamyl solution added to each sample. Samples were shaken by hand twice at 5 min intervals followed by a final centrifuge step (3, 800 RCF, 4 °C).

Four mL of each supernatant was removed and 8 mL of 95% ethanol was added to it. Samples were shaken by hand and placed in the freezer (-20°C) overnight to precipitate the DNA. Samples were centrifuged at room temperature until 3800 RCF was reached. The supernatant was decanted and the DNA pellets were left to dry for 10 min at room temperature prior to washing twice with 70% ethanol. Ethanol was then evaporated off by placing the samples in an incubator (37˚C) until dry. The pellets were then resuspended in 400 µL of sterile Milli Q water.

The samples were transferred to 2 mL Eppendorf tubes for long-term storage in the freezer. A 200 µL volume of each sample was transferred to a new 2 mL Eppendorf tube and cleaned using the Ultra Clean® 15 DNA Purification kit (MoBio Pty Ltd, Carlsbad, CA, USA). The manufacturer's protocol used was followed, adjusting the volumes for each buffer used where applicable. To each DNA sample, 15 µL of silica binding agent was used was added resulting in the final DNA volume of 30 µL. A 10 µL subsample of the cleaned DNA was cleaned again to remove PCR inhibitors using Ultra Clean (Bioline Life Sciences) (refer to Chapter Two, section 2.2.2).

A total of ninety-five DNA samples were obtained from the 96 juice samples. Preliminary quantification of a selection of samples using a Thermo Fischer nano drop 8000 found that DNA and other proteins were present in samples after initial DNA extraction (results not shown). This step was completed prior to the additional

cleaning steps using MoBio's Ultra Clean® 15 DNA Purification kit and Bioline's Ultra Clean kit. The extracted DNA samples were quantified using the Quant-iT PicoGreen Assay (Invitrogen, Australia) and the real-time PCR machine (RotorGene 6000) (formerly Corbett Life Sciences, now Qiagen Pty Ltd), following the manufacturer's recommendations. The quantification was performed using 3 µL of DNA sample, which was diluted with 47 µL of 1X TE buffer (Invitrogen, provided with kit). The diluted PicoGreen dye, which was made up as per the manufacturer's specifications, was then added to the diluted DNA samples. Quantification was performed in duplicate using 25 µL sub-samples of the diluted DNA containing the PicoGreen dye and the real-time qPCR machine was programmed to run the specific settings for the PicoGreen assay. Samples were quantified by comparison with a set of seven dilutions made up using the supplied calf thymus DNA stock and TE buffer (Quant-iT kit). Once quantified, samples with a concentration of DNA above 5 ng/µL, were normalised to this value using sterile Milli Q water.

The duplex qPCR assay previously developed (refer to Chapter 2, Section 2.2.5 for further detail) was used to quantify the amount of *B. cinerea* DNA present in each sample. Each reaction contained a final volume of 25 µL made up with 12.5 µL 2× StrataGene Brilliant® II qPCR Master Mix (StrataGene, Agilent Technologies, California, USA), 0.3 µM of each primer (KJD, BcF, BcR, GF, GR), 0.2 µM of each probe (KJD BcP, GP), 2.5 µL DNA sample and the rest of the volume was made up with sterile Milli Q water. Cycling conditions included an initial activation step of 95°C for 10 mins, followed by 40 cycles of 95°C for 30 s, 50°C for 1 min and an extra extension step of 72°C for 15 s. A RotorGene 3000 real-time PCR machine (formerly Corbett Life Sciences) was used to quantify and record the fluorescence data.

4.2.5.3. Juice quality assessment via mid-infrared spectroscopy

Following the advice of Dr Robert Dambergs (Australian Wine Research Institute), mid-infrared (MIR) spectroscopy was conducted on harvest-date juice samples from the field trial. A 1.5 mL volume per sample of the frozen juice was thawed and within 30 min of defrosting, the sample was vortexed for 20 s and then left on the bench for

approximately 30 mins to bring the juice sample to room temperature. Prior to testing, the samples were vortexed again to ensure adequate mixing of the sample. Mid-infrared (MIR) spectra were collected with a Bruker Alpha-P spectrophotometer, using a diamond attenuated total reflectance (ATR) sample presentation method. Prior to testing, the machine was allowed to warm up and calibrated with a blank of distilled water, and then re-calibrated every 10 samples. Using a Pasteur pipette the juice sample was mixed and approximately 500 μL was placed onto the diaphragm and the MIR spectra recorded. Data were acquired using the Bruker *Opus* software.

4.2.6. Data analysis

4.2.6.1. Field data

All statistical analyses including linear regression for the disease progress curves were completed using GenStat 10th edition, version 10.1 (VSN International Ltd, UK).

For the analysis of visual data obtained in the field, the dates on which the data were collected were transformed to a number using the Microsoft dating system starting at January 1, 1900. The disease severity data were logit transformed prior to analyses (Beresford *et al*. 2006) (Equation B6, Appendix B). Factorial Analysis of Variance (ANOVA) was used to determine significant differences between the fungicide treatments; trash removal and bunch position using mean values per replication both with and without transformation. Standard error (SE) and least significant difference were also calculated. ANOVA was also used to determine differences between treatments for vineyard data collected (vine characteristics and juice analysis). When factorial ANOVA could not be used due to uneven sample size, one-way and two-way ANOVA were used instead.

Temporal disease progression curves were generated using all visible disease severity data. Analysis of the area under the disease progression curve (AUDPC) was used, as this is a preferred method for analysing temporal disease data (Jeger and Viljanen-Rollinson 2001; Mohapatra *et al*. 2008). A repeated measure ANOVA was also performed on the severity and incidence data of BBR. Fitted values were calculated to produce a predictive model to determine the date at which the treatments would

reach 5% BBR severity. To calculate the date, back-transformation of the calculated fitted logit severity data was used (refer to equation B7 Appendix B) as described by Beresford *et al*. (2006) and Evans *et al*. (2010a).

4.2.6.2. Analysis of qPCR data

Data analysis was performed the software Rotor-Gene Q, Pure Detection (version 1.7, Build 94). Data sorting and summarising was completed using Microsoft Office Excel (Mac 2008, version 12.2.7) prior to statistical analysis. Reaction efficiencies (E) were calculated using the equation described by Bustin *et al*. (2009) (Equation B2, Appendix B). Regression analyses to check for run variation and ensure runs were not statistically different for the standard dilution series were performed using GenStat version 10.1. Standard errors were calculated for both the Ct values for the standards and the quantified amount of *B*. *cinerea* DNA in the juice samples.

4.2.6.3. Analysis of immunoassay data

Mean signal intensity (SI) readings and their standard errors (SE) were calculated for each treatment. Factorial Analysis of Variance (ANOVA) was performed on SI data to determine significant differences where $P = 0.05$. Least significant difference was also calculated (lsd). All statistical analyses were performed using GenStat version 10.1.

4.2.6.4. MIR data and principle component analysis

Principle component analysis (PCA) of MIR data relating to the juice samples was performed in consultation with Dr Robert Dambergs (AWRI) using *Systat* software, v10.0. The procedure was performed using *The Unscrambler*, version 9.8 (Camo, Norway) on untransformed spectra. Cross-validation was used during the calculations (20 groups with 5 samples per group) and the data was centred. The key parameters derived by the calculations were the principal component scores and the loadings to derive those scores. PCA was also used to determine if there were correlations between MIR readings, amount of *B*. *cinerea* DNA, SI and BBR severity and treatment differences. Data for severity, DNA amount and SI values were divided

into categories for further PCA analysis. DNA amount was grouped in very low (<0.01pg) low (0.01- 0.2 pg), medium (0.2- 1.0 pg), high (1-70 pg) and very high (>70 pg). SI values were grouped into similar categories regarding the SI value recorded for the sample. Categories were as followed: - low: SI >10, medium: 11-29; and high: ≥30. BBR severity was divided into four groups of varying percentage severity which included: - A) 0-2%; B) 2-4%; C) 4-6% and D) 6-13 %. Analysis of Variance (ANOVA) and Tukey pairwise analysis was used to determine if there were treatment differences in final disease severity, MIR, qPCR and ELISA data.

4.3. Results

4.3.1. Canopy and bunch trash as sources of inoculum

Incubation of the canopy and bunch trash (calyptras, aborted berries) failed to demonstrate evidence of significant colonisation by *B. cinerea*. A small number (approximately 0.005%) of calyptras and even fewer aborted berries contained *B. cinerea*. Other fungi observed colonising the bunch trash included species of *Alternaria*, *Penicillium* (0.04% calyptras; 0.02% aborted berries) and *Aspergillus* (0.007% calyptras; 0.01% aborted berries).

4.3.2. Expression of latent B. cinerea infection

4.3.2.1. Pre-bunch closure

After 9 days of incubation of the berries from treatments 1-4, there was no significant difference in *B. cinerea* incidence (mean incidence range 0.63- 3.44%) between treatments for both fungicide and trash removal (P = 0.222 and P = 0.385; respectively) (Refer to Table E1, Appendix E). However, after 13 days of incubation, the pea-size cyprodinil + fludioxonil treatment showed a significant reduction in incidence of *B. cinerea* compared with the nil treatment (Table 4.2). There was also a significant interaction between fungicide treatment and trash removal treatment, where both the pea-size spray and trash removal was applied resulted in the lowest incidence of latent *B. cinerea* infection.

Table 4.2: Mean *B. cinerea* percentage incidence (%) in ONFIT berries, with results of a factorial ANOVA after 13 days of incubation (residual df = 15). Treatments examined were that of nil and pea size fungicide application with either trash removal (Yes) or no trash removal (No). Analysis completed using logit transformed values (in brackets). The P values for each factor are as followed:- Fungicide *P* = 0.048 (lsd 0.968); trash removal *P* = 0.124; interaction *P* = 0.012 (lsd = 1.369 Means with the same letter are not significantly different at *P* = 0.05. Letters under the mean column correspond to the fungicide treatment only, while the other four correspond to the interaction between trash removal and fungicide.

Growth Stage	Fungicide	Trash Removal		Mean
		Yes	No	
Nil		13.5 (-1.92) a	11.0 (-2.48) a	12.3 (- 2.20) a
Pea	cyprodinil+fludioxonil	2.5 (-4.20) b	11.7 (-2.16) a	7.1(- 3.18) b
	Mean	8.0 (-3.06)	11.4 (-2.32)	

4.3.2.2. Véraison

Seven days of berry incubation post freezing demonstrated that all fungicide treatments (cyprodinil + fludioxonil at Pea size and PBC, and preharvest) significantly reduced the incidence of *B. cinerea* in comparison with the control treatment (P = 0.004, lsd = 0.996) (Table 4.3). The trash removal treatment had no effect and there was no significant interaction between fungicide and trash removal (Table 4.3). The fungicide treatments were not significantly different from each other with canopy trash removed, while there was variation for those without trash removed. By 14 days incubation post-freezing, the technique no longer discriminated between treatments with an increase in the expression of both *B. cinerea* and other fungi(data not shown). Other fungi that were identified (in low incidence) included *Aspergillus*, *Penicillium* and *Rhizopus* spp.

Table 4.3: Mean percentage (%) incidence of *B. cinerea* in ONFIT berries with results of a factorial ANOVA after seven days of incubation (residual df = 35). Samples were taken at *véraison* and included all fungicide treatments. *P* values from ANOVA as follows with calculated least significant values (lsd) in brackets:- fungicide *P* = 0.004 (lsd = 0.996); trash removal *P* = 0.761; interaction *P* = 0.087. The letter following the mean values for each treatment, when the letter following the mean is different from another, the treatments are significantly different (P ≤ 0.05).

Growth Stage	Fungicide	Trash removal		Mean
		Yes	No	
Nil		5.83 (- 2.86)	6.67 (- 2.69)	6.25 (- 2.77) a
Pea	cyprodinil+fludioxonil	2.50 (- 4.61)	2.78 (- 3.78)	2.64 (- 4.19) bc
PBC	cyprodinil+fludioxonil	2.92 (- 3.81)	0.97 (- 5.46)	1.94 (- 4.64) bc
Ver + 3wks	trifloxystrobin	3.06 (- 4.12)	3.47 (-3 .01)	3.26 (- 4.02) b
	Mean	3.58 (- 3.85)	3.47 (- 3.96)	

4.3.3. Expression of BBR in the field at harvest

Results for total BBR severity are presented here, whereas the results for the other BBR symptoms are shown in Appendix E (Table E2).

4.3.3.1. BBR severity

The 'nil' or 'no fungicide' treatment (1 & 2) resulted in the highest mean value for BBR severity and the trifloxystrobin treatment (7 & 8) had the lowest (Table 4.4) (Figure 4.1 for example of BBR), although treatments were not separated statistically (P = 0.065, residual df = 75) (Table 4.4). There was no effect of trash removal (P = 0.794) (Table 4.5).

The incidence of sporulating berries in the bunch was significantly reduced by all fungicide treatments (P = < 0.001, residual df = 75) (Table 4.5). Once again, trash removal had little effect (P = 0.672, 0.141 respectively, residual df = 75) (Table 4.5). Bunch position (basal or distal) also had no effect.

Some of the block was highly exposed to the sun resulting in sunburn to 20 - 40% of the bunch (Figure 4.2). Other bunch rots identified during the assessments included were caused by *Penicillium* and *Aspergillus* spp. and *Colletotrichum acutatum*. Some bunches contained split berries, allowing rots to become established (Figure 4.3).

Table 4.4: Mean total BBR severity (%) at harvest with results of a factorial ANOVA (P = 0.05, residual df = 75). Analysis was completed using logit-transformed data (in brackets). P values for each treatment are as follows: - fungicide P = 0.065; trash removal P = 0.794; interaction P = 0.145.

Growth Stage	Fungicide	Trash removal		Mean
		Yes	No	
Nil		4.81 (- 3.15)	3.58 (- 3.42)	4.20 (- 3.28)
Pea	cyprodinil+fludioxonil	3.26 (- 3.46)	3.17 (- 3.54)	3.22 (-3.50)
PBC	cyprodinil+fludioxonil	2.44 (- 3.75)	3.39 (- 3.36)	2.92 (- 3.55)
Ver + 3wks	trifloxystrobin	2.41 (- 3.71)	2.85 (- 3.64)	2.63 (- 3.68)
	Mean	3.23 (- 3.52)	3.25 (- 3.49)	

Table 4.5: Mean percentage (%) severity of sporulating *B. cinerea* at harvest showing results from a factorial ANOVA (*P* = 0.05, residual df = 75). Analysis was conducted using logit-transformed values (shown in brackets). Calculated P values from ANOVA are as followed with least significant differences values in brackets (lsd):- fungicide *P* = <0.001 (lsd = 0.3429); trash removal *P* = 0.672; interaction *P* = 0.141 Least significant difference is also shown (lsd) *P* = ≤ 0.05. Values with same letter are not significantly different

Growth Stage	Fungicide	Trash removal		Mean
		Yes	No	
Nil		0.04 (- 5.62)	0.36 (- 5.59)	0.20 (- 5.61) a
Pea	cyprodinil+fludioxonil	0.10 (- 6.39)	0.08 (- 6.48)	0.09 (- 6.43) b
PBC	cyprodinil+fludioxonil	0.02 (- 6.77)	0.20 (- 6.25)	0.11 (- 6.51) b
Ver + 3wks	trifloxystrobin	0.11 (- 6.44)	0.03 (- 6.70)	0.07 (- 6.57) b
	Mean	0.07 (- 6.31)	0.17 (- 6.26)	

Figure 4.1: Pink brown rot characteristic of BBR with some sporulation (refer to blue arrow pointing to the sporulation). There are some signs of other bunch rots within the bunch.

Figure 4.2: Example of severe sunburn damage observed in sections of the trial site.

Figure 4.3: Split berries that gradually shrivelled up (refer to grey arrow in figure pointing to the splitting).

.

4.3.3.2. BBR incidence

During the season, BBR incidence was generally low with little difference between treatments. For example, the assessments made on the 2nd April showed that incidence was not significantly different for either fungicide or trash removal treatments ($P = 0.719$ and $P = 0.278$, respectively) (Table E3, Appendix E). On the final assessment (7th April 2008) just prior to harvest, however, there was a marked increase in BBR incidence and the effect of fungicide timing was highly significant ($P < 0.001$) (Table 4.6). Trash removal had little effect on its own, but there was a significant interaction between the fungicide and trash removal ($P = 0.007$) (Table 4.6). Cyprodinil + fludioxonil applied at PBC and trifloxystrobin applied post-*véraison* reduced BBR incidence at harvest (7th April, 2008) compared with cyprodinil + fludioxonil applied at Pea-size and the nil treatment.

Table 4.6: Mean percentage incidence (%) of BBR at harvest (8/04/08) with results from a a factorial Analysis of Variance (residual df = 35). ANOVA P values for each factor is as followed with the least significant difference value in brackets (lsd): - fungicide *P* = <0.001 (7.195); trash removal *P* = 0.267; interaction *P* = 0.007 (10.175). Numbers with the different letter shown significant difference. The letters under the Mean column correspond with fungicide means only. The letters in the trash removal columns show the difference corresponding with the interaction between fungicide and trash removal.

		Trash Removal		
Growth Stage	Fungicide	Yes	No	Mean
Nil		75.00	77.38	76.19 a
Pea	cyprodinil+fludioxonil	79.76	67.26	73.51a
PBC	cyprodinil+fludioxonil	58.33	69.64	63.99 b
Ver + 3wks	trifloxystrobin	57.74	67.86	62.80 b
	Mean	67.71	70.54	

4.3.3.4. Severity of harvested bunches pre and post incubation

After incubation of the selected bunches (6 bunches each treatment replication), the mean severity of harvested BBR increased significantly (Table 4.7 & 4.9; Table E4, Appendix E). The nil treatments had the highest severity and sporulation relative to the other treatments. For total BBR, similar results were obtained with the cyprodinil + fludioxonil treated vines (treatments 3-6) resulting in lower mean severity levels than the control both before (1.56% - 4.86%) and after incubation (2.58- 5.61%), with the PBC (treatments 5 and 6) treatment being the most effective ($P = 0.27$ and 0.001

respectively) (Table 4.7, 4.8). No significant interaction was found between fungicide treatment and trash removal (Table 4.7 &4.8) or bunch position (data not shown).

The amount of sporulating *B*. *cinerea* ranged from 0.03 – 0.5 % at harvest, but after incubation there was an increase in all treatments (ranging from 0.17- 8.58 %). After incubation, the significance of the difference between the nil treatment and the fungicide treatments increased, with the *P* value decreasing from 0.031 to < 0.001 (Table 4.9. 4.10). However there was no significant effect of for trash removal or a significant correlation between it and the fungicide treatments. Other rots observed in the bunches included *Penicillium* spp.

Table 4.7: Mean total BBR severity prior to incubation with results from a factorial ANOVA using logit-transformed values (in brackets) (residual df = 95). ANOVA *P* values for each factor are as follows with the least significant difference value in brackets (lsd): - fungicide *P* = 0.027 (0.638); trash removal *P* = 0.345; interaction *P* = 0.112. LSD in table is represented by a letter, means with a difference letter signify a significant difference. Values with same letter are not significantly different

Growth Stage	Fungicide	Trash Removal		Mean
		Yes	No	
Nil		5.06 (- 3.52)	3.13 (- 3.73)	4.10 (- 3.63) a
Pea	cyprodinil+fludioxonil	4.78 (- 3.16)	4.29 (- 3.63)	4.55 (- 3.40) a
PBC	cyprodinil+fludioxonil	1.56 (- 4.56)	3.44 (- 3.70)	2.50 (- 4.13) b
Ver + 3wks	trifloxystrobin	1.61 (- 4.59)	3.39 (- 3.92)	2.50 (- 4.25) b
	Mean	3.39 (- 3.96)	3.56 (- 3.74)	

Table 4.8: Mean total BBR severity (%) after incubation with results from an factorial ANOVA (df = 95). Severity was logit-transformed prior to analysis (in brackets). *P* values from the ANOVA are as follows with least significance difference (lsd) in brackets: - fungicide *P* = 0.001 (0.726); trash removal *P* = 0.613; interaction *P* = 0.445 The lsd is represented in table by letters, where the means with the same letter are not significantly different.

Growth Stage	Fungicide	Trash removal		Mean
		Yes	No	
Nil		11.56 (- 2.29)	10.06 (- 2.54)	10.81 (- 2.41) a
Pea	cyprodinil+fludioxonil	4.25 (- 3.40)	5.61 (- 3.72)	4.93 (- 3.56) b
PBC	cyprodinil+fludioxonil	2.58 (- 3.98)	5.22 (- 3.53)	3.90 (- 3.76) b
Ver + 3wks	trifloxystrobin	3.44 (- 3.88)	7.33 (- 3.23)	5.39 (- 3.55) b
	Mean	5.46 (- 3.39)	7.06 (- 3.26)	

Table 4.9:Mean percentage (%) of sporulating *B. cinerea* in harvested bunches prior to incubation. A factorial ANOVA was performed using logit-transformed values (in brackets) (*P* = 0.05, df = 95). *P* values from the ANOVA are as follows with least significance difference (lsd) in brackets: - fungicide *P* = 0.031 (0.4758); trash removal *P* = 0.781; interaction *P* = 0.703. The lsd is represented in table by letters, where the means with the same letter are not significantly different.

Growth Stage	Fungicide	Trash removal		Mean
		Yes	No	
Nil		0.28 (- 5.90)	0.50 (- 6.19)	0.39 (- 6.04) a
Pea	cyprodinil+fludioxonil	0.08 (- 6.62)	0.17(- 6.57)	0.13 (- 6.58) b
PBC	cyprodinil+fludioxonil	0.03 (- 6.79)	0.11 (- 6.57)	0.07 (- 6.68) b
Ver + 3wks	trifloxystrobin	0.17 (- 6.55)	0.06 (- 6.74)	0.12 (- 6.642) b
	Mean	0.28 (- 6.46)	0.42 (- 6.51)	

Table 4.10: Mean percentage of sporulating *B. cinerea* in harvested bunches after incubation (%). A factorial ANOVA using logit-transformed percentage (in brackets) was completed (df = 95). *P* values from the ANOVA are as follows with least significance difference (lsd) in brackets: - fungicide *P* = <0.001 (0.811); trash removal *P* = 0.959; interaction *P* = 0.367. The lsd is represented in table by letters, where the means with the same letter are not significantly different.

Growth Stage	Fungicide	Trash removal		Mean
		Yes	No	
Nil		8.58 (- 3.43)	5.33 (- 3.96)	5.58 (- 3.69) a
Pea	cyprodinil+fludioxonil	0.81 (- 5.84)	0.53 (- 6.16)	0.67 (- 6.00) b
PBC	cyprodinil+fludioxonil	0.17 (- 6.44)	1.53 (- 5.62)	0.85 (- 6.03) b
Ver + 3wks	trifloxystrobin	0.14 (- 6.37)	0.39 (- 6.27)	0.27 (- 6.32) b
	Mean	2.41 (- 5.52)	1.95 (- 5.50)	

4.3.4. Temporal progression

There was no significant effect of fungicide treatment on AUDPC for total BBR severity (Table 4.11). Repeated measures ANOVA of total BBR severity showed that time was a significant factor (Table 4.11), but none of the fungicide treatments, trash removal or interactions were significant. The slight decrease observed on the 12th March may have resulted from diseased berries dropping off the vine (Figure 4.4). It was noted at this stage that botrytised berries were starting to shrivel and dry up. The late increase can be attributed to a late rainfall event prior to harvest (refer to section 4.3.6). Regression curves generated using the logit-transformed values were significant for each of the treatments (Table 4.11).

Table 4.11: Linear Regression Analysis for temporal progression of total BBR for each of the treatments using logit transformed data. The AUPDC was calculated using mean actual severity scores per treatment and replication.

Trt	Description	n^2	Slope	Intercept	*P* Value	R^2 (adj)	AUDPC
1	Nil + Trash Removal	5	0.1022	- 4047	0.035	0.640	17.22
2	Nil	5	0.0948	- 3754	0.035	0.638	17.06
3	Pea size + Trash Removal	5	0.0965	- 3820	0.035	0.637	12.41
4	Pea size	5	0.0917	- 3631	0.038	0.623	12.35
5	PBC + Trash Removal	5	0.0877	- 3472	0.041	0.612	10.31
6	PBC	5	0.0999	- 3957	0.022	0.712	14.81
7	Ver + 3wks + Trash Removal	5	0.0862	- 3414	0.011	0.796	12.01
8	Ver + 3wks	5	0.0951	- 3764	0.023	0.702	13.10

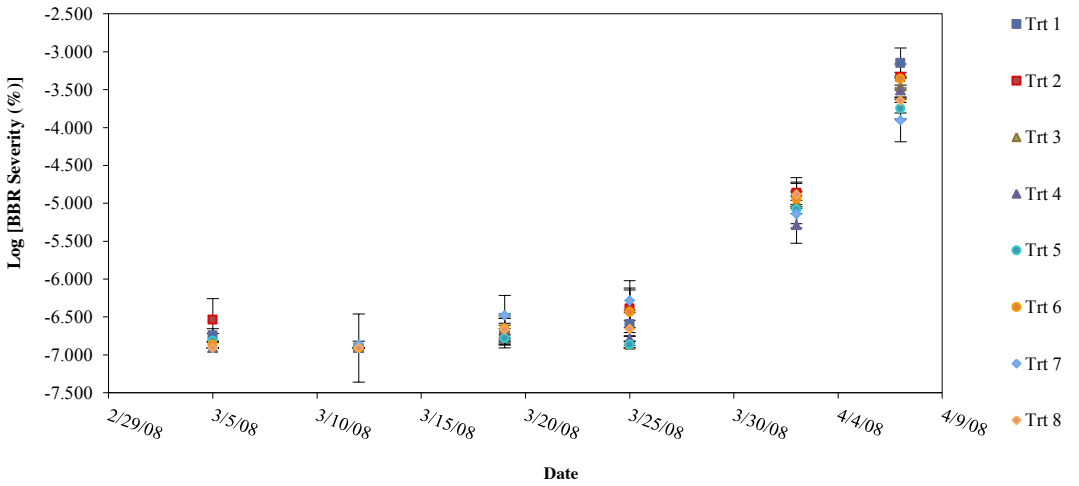

Figure 4.4: Temporal progression of total BBR severity using logit transformed values of percentage infection for all treatments. Standard error is also shown as error bars. The treatments are as follows:- Trt 1 – nil fungicide with trash removal; Trt 2 – nil fungicide only; Trt 3 – pea- size fungicide with trash removal; Trt 4 – pea-size fungicide only; Trt 5 fungicide at PBC with trash removal; Trt 6 PBC fungicide only; Trt 7 fungicide at *véraison* + 3wks later with trash removal; Trt 8 fungicide at *véraison* + 3wks later only.

Table 4.12: Repeated measures analysis of variance showing the effect of time on progression of total BBR severity. The data were log transformed.

Source	df	SS	MS	F value	P > F
Fungicide	3	2.3132	0.7711	1.49	0.224
Trash Removal	1	0.2393	0.2393	0.46	0.499
Fungicide. Trash	3	0.9747	0.3249	0.63	0.599
Residual	83	42.9769	0.5178	2.17	-
Time	5	905.4911	181.0982	757.89	< 0.001
Time. Fungicide	15	5.5465	0.3698	1.55	0.109
Time. Trash Removal	5	0.2580	0.0516	0.22	0.924
Time. Fungicide. Trash Removal	15	3.9907	0.2660	1.11	0.349
Residual	440	105.1383	0.2390	-	-
Total	575	1069.4907	-	-	-

4.3.4.1. Disease prediction

Using the linear regression analysis for total BBR (as shown in Table 4.13), predictive dates based on fitted values were calculated at which the different fungicide treatments would reach the 5% threshold for BBR severity. Based on low visible *B. cinerea* observed at each of the assessment dates, the disease prediction model predicted dates for the severity of BBR to reach 5% well beyond the harvest date (9th April) for all treatments given the grapes were destined for table wine. The earliest predicted date to reach the 5% threshold for total BBR was the 15th April for treatment 6 (PBC with no trash removal) (Table 4.13). The earliest calculated date for the nil fungicide treatments was the 17th April (Table 4.13 and Figure 4.5). The latest date was the 28th April for treatment 7 (trifloxystrobin with trash removal), followed by treatment 4 (Pea-size without trash removal) and 5 (PBC with trash removal).

Table 4.13: Epidemic prediction for total BBR for each of the treatments based on the data presented in table 4.12. The 5% severity columns show the calculated actual date at which each of the treatments would reach 5% total BBR with the rate of increase to obtain it (based on the methodology of Beresford *et al.* (2006)). The Actual Harvest column is the calculated severity at which the treatments would reach on the actual harvest date and the actual increase rate to reach it.

Trt	Actual Harvest	5 % Severity		Actual Harvest	
		Date	Increase Rate at 5% Severity	Severity at Harvest	Increase rate
1	9th April 2008	19th April 2008	0.5181	1.71	0.19
2		17th April 2008	0.4791	2.19	0.22
3		17th April 2008	0.4879	2.18	0.23
4		26th April 2008	0.4595	0.98	0.10
5		24th April 2008	0.4415	1.31	0.13
6		15th April 2008	0.5062	2.63	0.28
7		28th April 2008	0.4338	0.89	0.09
8		21st April 2008	0.4803	1.47	0.15

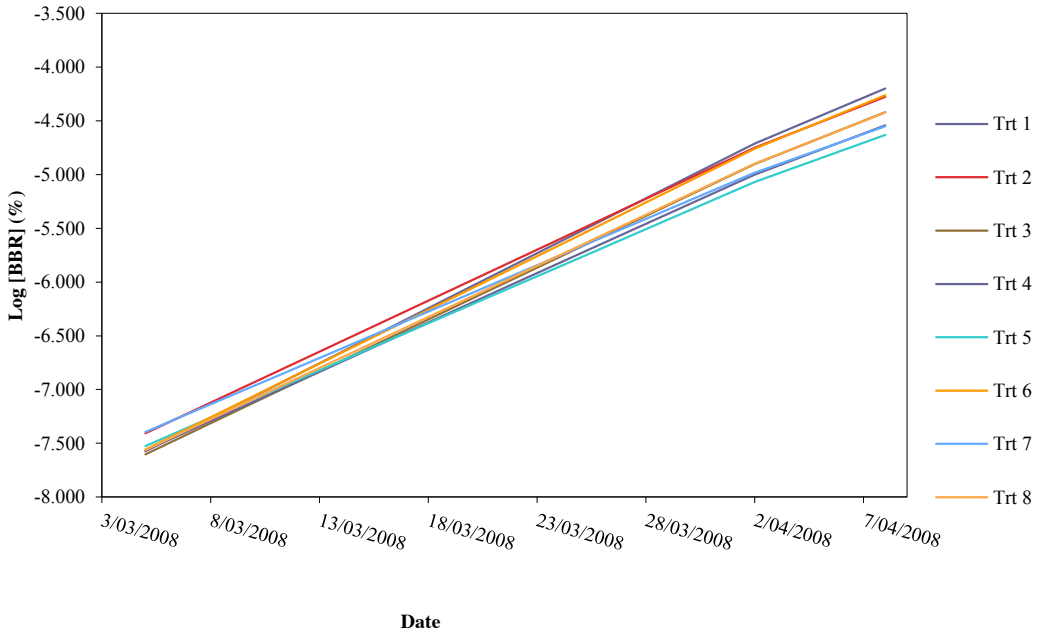

Figure 4.5: Fitted logit total BBR severity values used to derive regression parameters based on the data presented in Table 4.14 and Figure 4.6. BBR severity was transformed as per Beresford *et al.* (2006).

4.3.5. Bunch compactness

Mean bunch compactness was in the middle of the range (where 0 = compact and 100 = loose) (Table 4.14) with no significant difference between replicates (each replicated was a separate row) (P = 0.659). There was a significant difference between bunch position (P = 0.009), with basal bunches (B) being more compact than

distal bunches (D) (Table 4.14), but there was no significant interaction between replicates and bunch position ($P = 0.074$, total df = 47). Mean total BBR severity for the nil fungicide treatment suggested that there was a relationship with bunch position, since the more compact basal bunches had higher BBR severity than the distal bunches (Table 4.14). However bunch position was found to not be a significant factor for BBR severity according to the results of the ANOVA (analysis not shown).

Table 4.14: Mean bunch compactness (%) separated into replication (row) and bunch position (basal (B) and distal (D)). Mean compactness for trial site is also shown and standard error (SE) is shown. Mean BBR severity is shown for the nil fungicide treatment for bunch position.

Bunch Position	1	2	3	4	5	6	Mean	BBR Severity (%)
B	52.81 (6.88)	67.77 (6.88)	72.39 (3.90)	64.20 (3.02)	64.45 (4.93)	71.31 (3.59)	65.49	4.93
D	65.82 (3.49)	59.22 (7.10)	60.26 (3.50)	52.04 (3.44)	52.96 (11.39)	48.31 (3.23)	56.44	3.46
Mean	***59.32***	***63.49***	***66.33***	***58.12***	***58.71***	***59.81***	***60.96***	***-***

4.3.6. Analysis of grape juice / must

Total soluble solids measured during the season

The total soluble solids increased as the grapes ripened during the season, as expected (Figure 4.6), although there was a slight decrease and/p revelling between 20 -30th March 2008. This correlated with rain events that occurred during this period (refer to Section 4.3.11). Linear regression analysis found that there was no significant difference between the replications for this juice character.

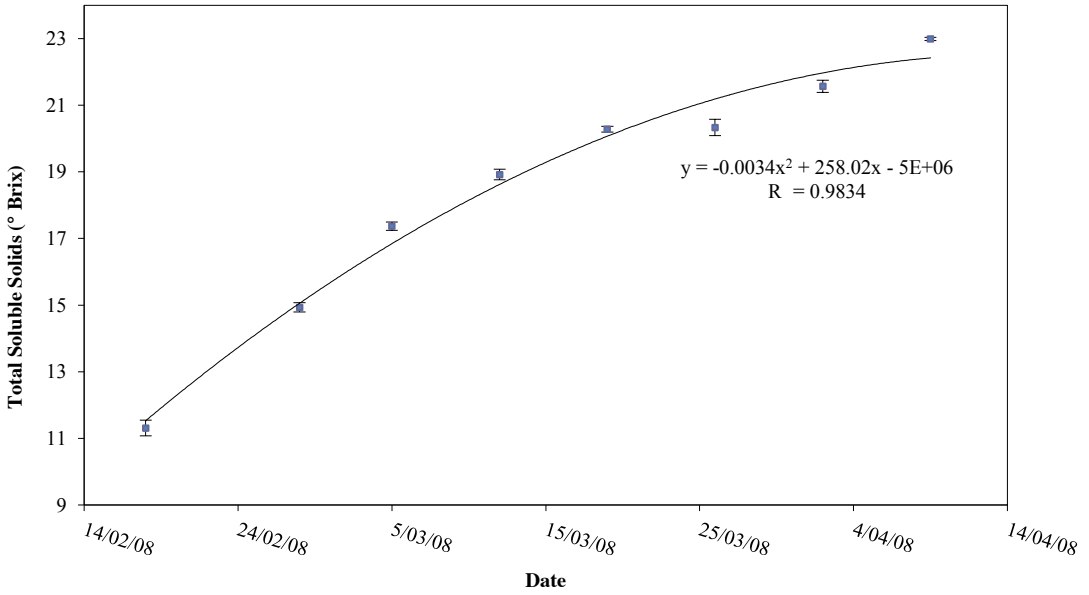

Figure 4.6: The increase of TSS (°Brix) in grapes for the trial site during ripening until harvest. Standard error (SE) is shown as error bars.

4.3.7. DNA extraction

All juice samples yielded between 1 and 3 ng/μL DNA.

4.3.8. Application of qPCR in determining amount of B. cinerea DNA

4.3.8.1. Standard dilution series

Linear regression analysis showed that the standard curves for each of the qPCR runs were not significantly different from each other (P =>0.05). The reaction efficiencies calculated for each of the qPCR runs ranged from 83.29 to 98.97 % (Figure E1 and Table E4 in Appendix E). The R^2 for each of the runs were either 0.98 or 0.99, which is above the minimum acceptable limit of 0.95. A linear regression was generated using the concentration of the standards and their respective Ct value for each run, to calculate the amount of *B. cinerea* DNA detected in the juice samples.

4.3.8.2. Juice samples

The qPCR assay detection limits in the juice samples were from 22 fg up to 0.522 ng DNA per reaction. *Botrytis cinerea* DNA was detected in 93 out of the 95 samples tested, and *V. vinifera* DNA was detected in all samples, giving confidence in the results. ANOVA showed no significant difference in the amount of *B. cinerea* DNA detected between treatments (Table 4.15). Both the nil and trifloxystrobin treatments had the highest mean amount of *B. cinerea* DNA while the two mid-season sprays (Pea and PBC) had the least. Analysis of the data according to trash removal suggested a trend, in which the removal resulted in lesser amounts of *B. cinerea* DNA in all treatments except trifloxystrobin (Table 4.15). In the detection of *V. vinifera* DNA (control), all samples resulted in similar Ct values (Table 4.15). The control was not used for quantitative purposes but rather qualitative to ensure PCR inhibition was not the cause of negative results.

Table 4.15: Summary of qPCR results for the juice samples tested for the amount of *B. cinerea* DNA. Mean Ct values and amount of *B. cinerea* DNA is shown with calculated standard error. The mean Ct value obtained for the *V. vinifera* grape control (Grape Ct) is also shown with calculated standard error. Refer to Table 4.18 for BBR severity.

Trt	Fungicide	Trash Removal	BC Ct	Mean DNA (pg)	Grape Ct
1	Nil	Yes	33.59 (0.69)	4.33 (1.73)	30.11 (0.53)
2		No	33.09 (0.67)	9.19 (5.88)	29.55 (0.54)
3	Pea	Yes	30.11 (2.84)	8.17 (3.95)	29.46 (0.63)
4		No	34.45 (0.67)	2.67 (1.16)	29.93 (0.44)
5	PBC	Yes	31.08 (2.94)	6.76 (3.84)	29.00 (0.44)
6		No	33.67 (0.99)	9.23 (4.55)	30.98 (0.92)
7	Flint	Yes	32.45 (0.94)	12.23 (5.93)	29.46 (0.56)
8		No	33.57 (1.10)	46.19 (43.29)	29.58 (0.50)

4.3.9. Application of QuickStix™ test

The results from the QuickStix™ test were found to discriminate treatment differences where the visual severity was unable to (P = 0.001) (Tables 4.16 and 4.17). Fungicide treatment significantly affected the SI (signal intensity) value. Both nil fungicide treatments (yes/no trash removal) had significantly higher SI values than the rest of the treatments (Table 4.18). Cyprodinil+ fludioxonil applied at pea-size (Treatments 3 and 4) had the lowest SI value (15.2), followed by PBC (Treatments 5 and 6), and then trifloxystrobin (Treatments 7 and 8). There was a significant interaction between fungicide and trash removal treatment (P = 0.047) (Table 4.18). Treatment 8 (trifloxystrobin only) had the lowest SI scores overall, even though it was sprayed with a fungicide that does not target BBR. There was no relationship found for bunch position (data not shown).

Table 4.16: Mean total BBR severity for harvested bunches used for juice analysis (SI and qPCR). Results from a factorial ANOVA is shown, severity values were logit transformed prior to analysis (P = 0.05, residual df = 75). The calculated P values from the ANOVA are as follows:- fungicide P = 0.313; trash removal P = 0.874 and interaction P = 0.377.

		Trash removal		
Growth Stage	Fungicide	Yes	No	Mean
Nil		4.81 (- 3.45)	3.58 (- 3.40)	4.20 (- 3.43)
Pea	cyprodinil+fludioxonil	3.26 (- 3.65)	3.17 (- 3.90)	3.21 (- 3.78)
PBC	cyprodinil+fludioxonil	2.44 (- 3.78)	3.39 (- 3.34)	2.92 (- 3.54)
Ver + 3wks	trifloxystrobin	2.41 (- 3.53)	2.85 (- 3.63)	2.63 (- 3.58)
	Mean	3.23 (- 3.59)	3.25 (- 3.57)	

Table 4.17: Mean signal intensity (SI) values from the QuickStix™ test of grape juice with results from a factorial ANOVA (P = 0.05, residual df = 75). The calculated P values and least significant differences (lsd) are as follows:- fungicide P = <0.001 (7.29); trash removal P = 0.203; interaction P= 0.047 (10.31). Letters shown in table represent the lsd where values with same letter are not significantly different.. The letters in the columns for trash removal show the lsd ranking concerning the interaction between fungicide and trash removal. The lsd ranking is also shown with regards to the effect of fungicide treatment in the mean column,

		Trash removal		
Growth Stage	Fungicide	Yes	No	Mean
Nil		42.2	38.7	40.4 a
Pea	cyprodinil+fludioxonil	15.8	13.4	14.6 c
PBC	cyprodinil+fludioxonil	18.7	25.6	22.2 b
Ver + 3wks	trifloxystrobin	30.2	16.0	23.1 b
	Mean	26.7	23.1	

4.3.10. Mid infrared spectroscopy and PCA analysis

The MIR spectra were determined for all samples, showing increased absorbance in the 950-1150 nm range (Figure 4.7). Of the 95 samples analysed, one sample was deemed an outlier and subsequently removed from the dataset for Principle Component Analysis (PCA) as the fluorescence reading was unusually high and resulted in the data being skewed. The PCA for the juice samples was unable to significantly distinguish between the treatments using the spectroscopy data (PC1 and treatment, $P = 0.099$) (Figure 4.8). The PCA analysis found no significant clustering using the categorised *B. cinerea* DNA amount for each samples and the spectral data obtained from the juice ($P = 0.646$) (Figure 4.9). Similar results were also obtained using the SI values and the spectral data ($P = 0.606$) (Figure 4.10). The Tukey pairwise analysis showed that the BBR severity for the samples with the higher SI scores were found to have a higher correlation value (highly correlated) than that of the samples which had low SI scores ($P = 0.01$) (Figure 4.11). There was also a correlation with the PC1 data from the MIR and the categorised visual BBR severity ($P = 0.001$) (Figure 4.12). The Tukey pairwise analysis showed that the PC1 values for BBR severity category A (0 – 2%) was significantly different from that of category B (2- 4%)($P = 0.035$) and category D (4 – 6%)($P = 0.001$) (Figure 4.13).

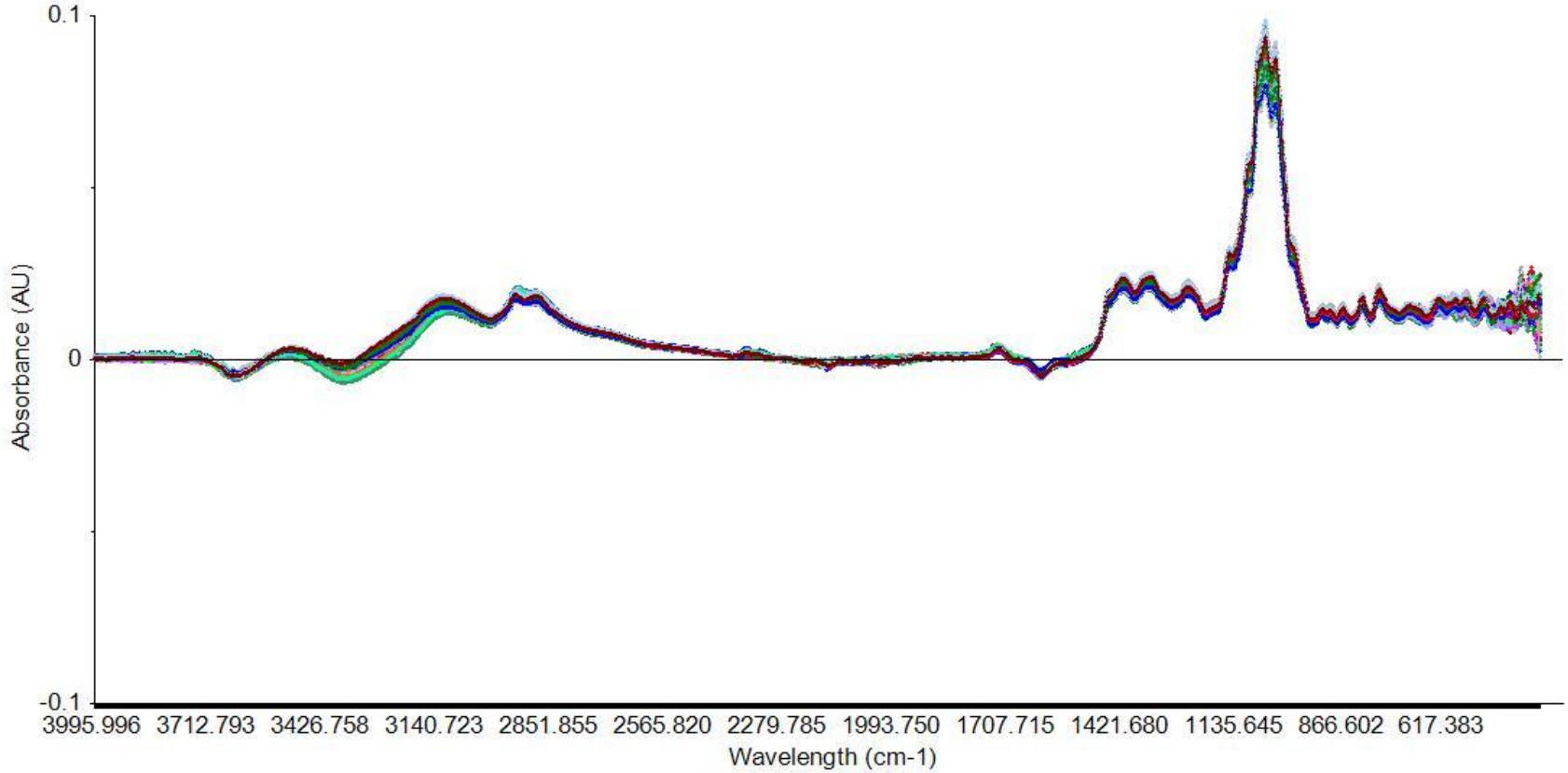

Figure 4.7: Raw data showing the wavelengths (x axis) and the absorbance (y axis) values for the juice samples examined. One sample was removed as it gave an unexplained higher absorbance.

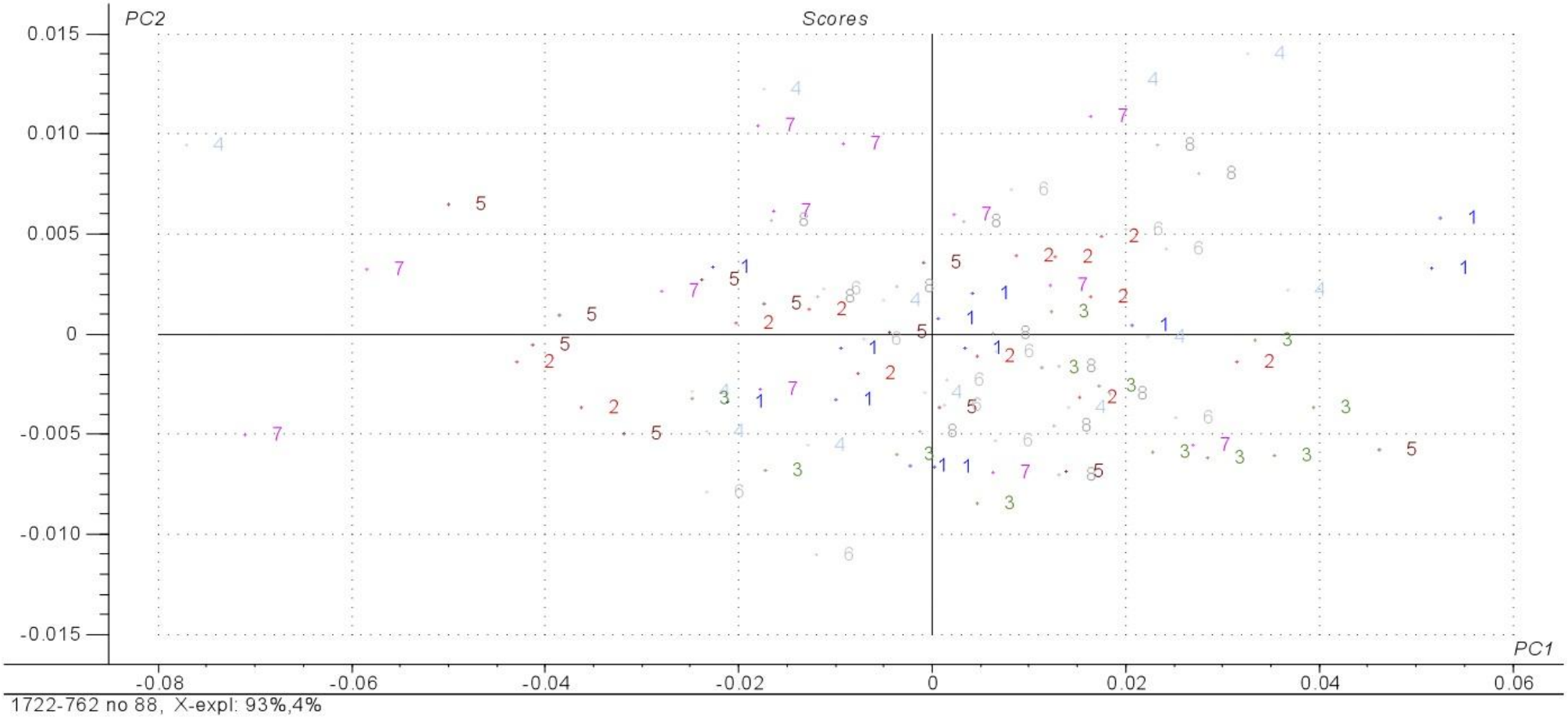

Figure 4.8: PCA analysis for treatment (treatments 1 - 8) differences using the MIR data (refer to Table 4.1 for treatment descriptions) (P = 0.099).

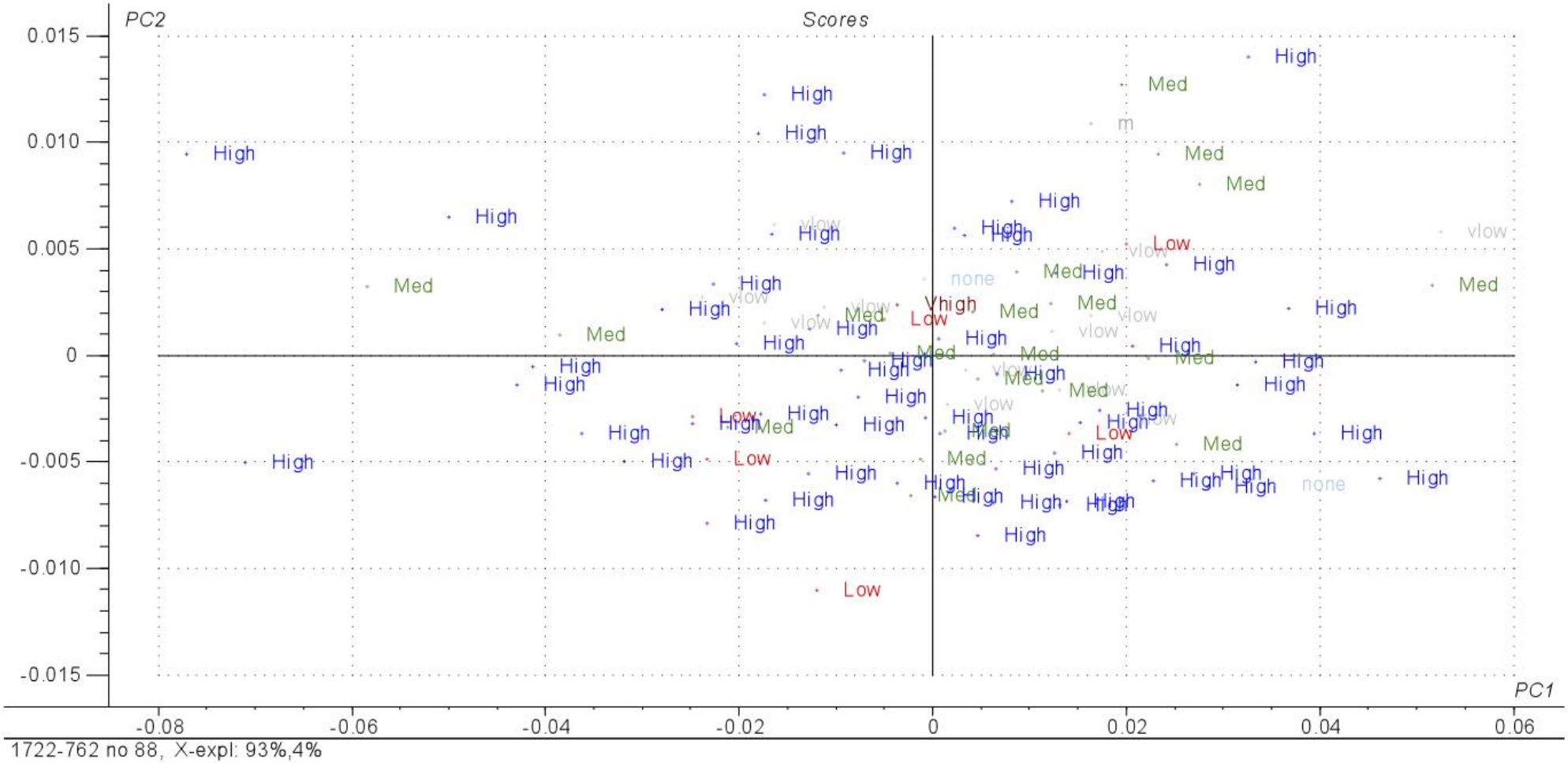

Figure 4.9: PC1 and DNA category (*P* = 0.646). DNA category ranged from very low, low to very high. Categories are as followed:- vlow = ≥ 0.01pg; low = 0.01 pg- 0.2 pg ; Med 0.2- 1 pg High = 1- 70 pg and Vhigh = ≥70 pg; none= no amplification/missing.

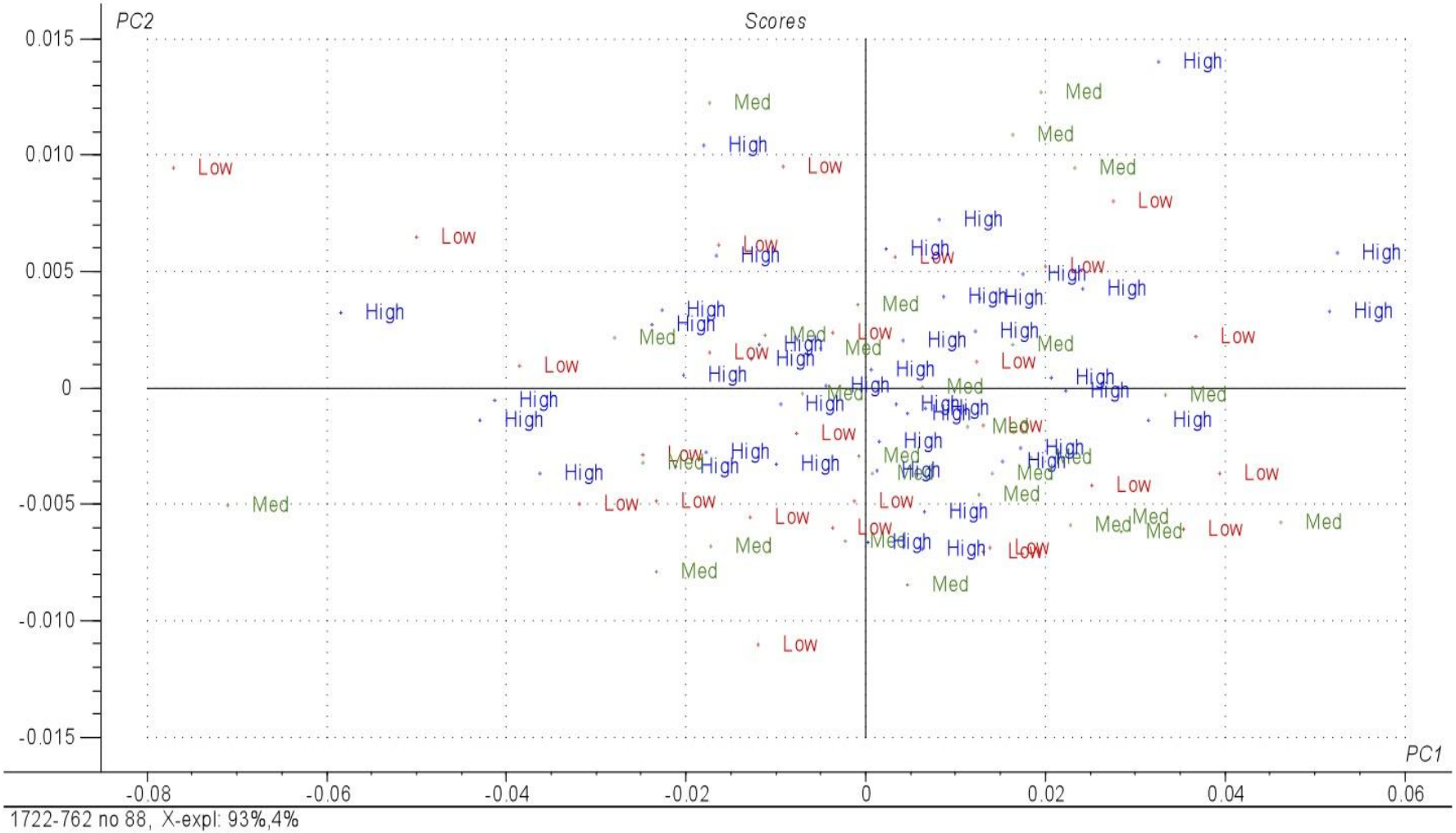

Figure 4.10: PCA Analysis for SI categories (QuickStix™ test) and MIR readings (*P* = 0.606). SI Values (categorised: Low = ≥10, Medium = 11- 30 and High = ≥ 30).

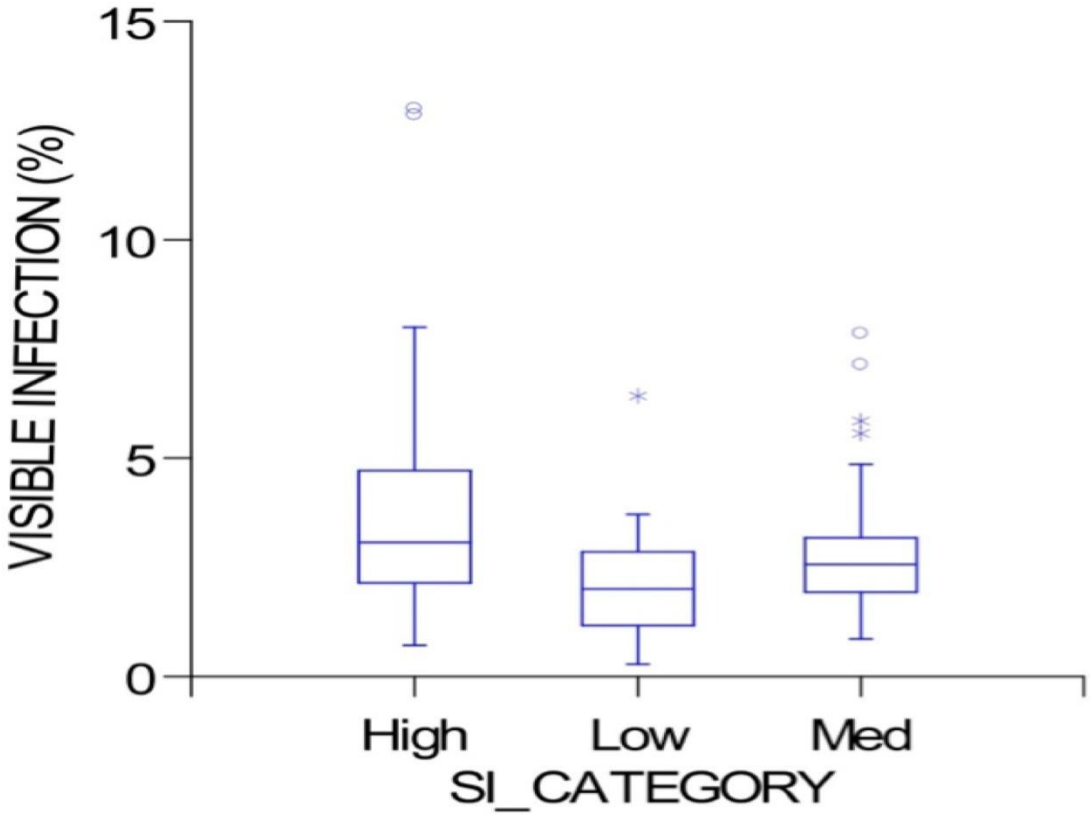

Figure 4.11: Tukey Pairwise analysis: high to low comparison for visible severity of BBR and Quick Stix™ SI Values (categorised: Low = ≥10, Medium = 11- 30 and High = ≥ 30) (*P* = 0.01).

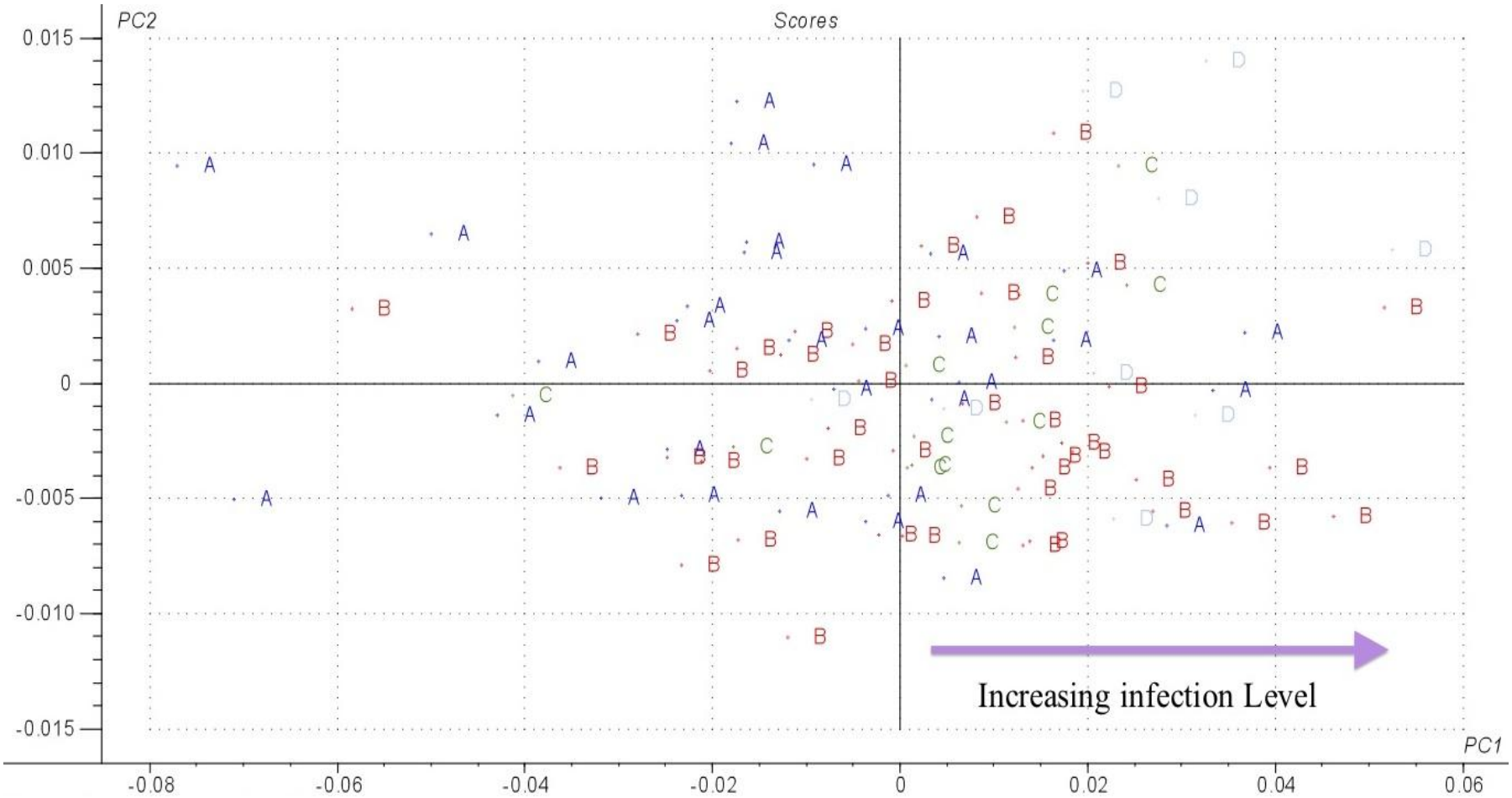

Figure 4.12: PCA results showing the samples grouped according BBR severity (x axis) and MIR PC1 readings (y axis) (ANOVA, *P* = 0.001). BBR severity categories as follows:- A = 0 – 2 %, B = 2 – 4 %, C = 4 – 6 % and D = 6 – 13 % BBR.

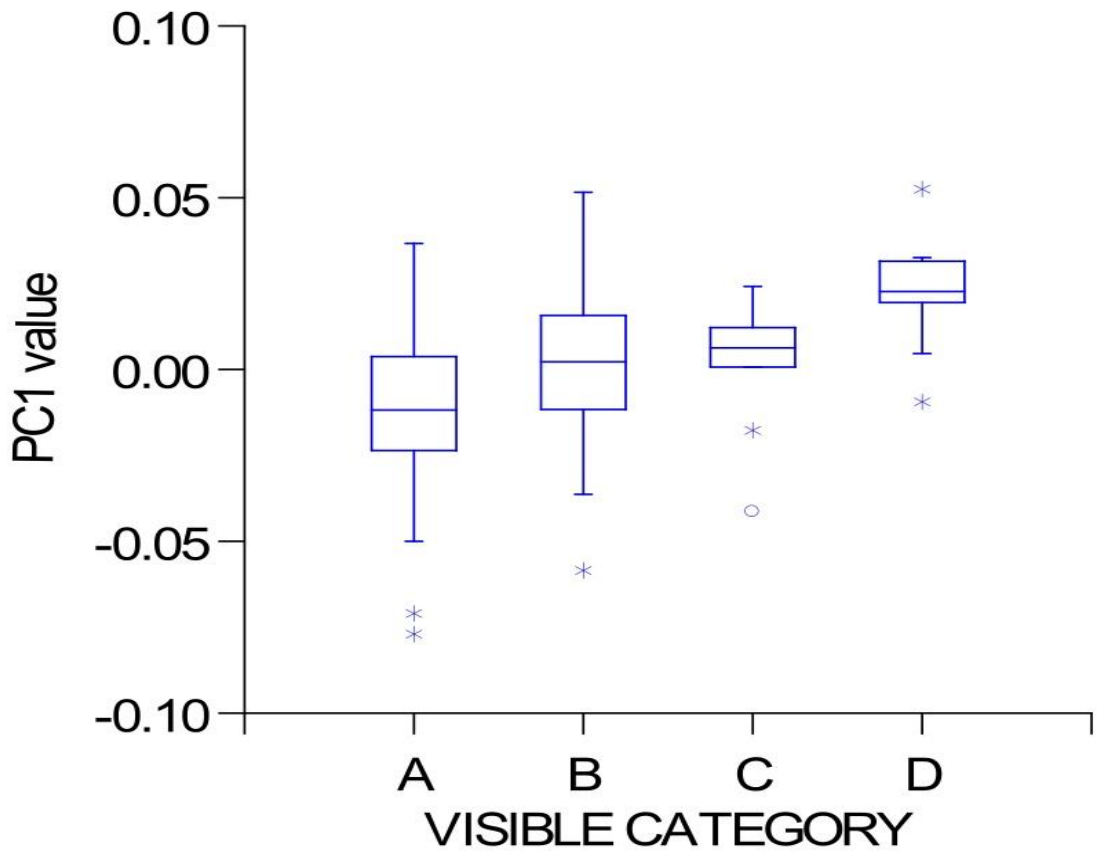

Figure 4.13: Tukey pairwise analysis for visible BBR severity category and PC1 value (A_B: P = 0.035 and A_D: *P* = 0.001). PC1 value is assigned to a sample, in this case the PC1 value is for the results from the MIR. Categories are as follows: A = 0 - 2%; B = 2 - 4%; C = 4 - 6% and D = 6 - 12%.

4.3.11. Environmental Data

Overall the season was not very conducive to the development of *B. cinerea*, which resulted in the BBR severity barely reaching the 5% threshold by harvest for any treatment (nil fungicide = 4.81%). There were only a few rainfall events above during the season, all followed by periods of dry weather limiting free moisture in the environment and keeping the mean relative humidity below optimum for BBR development (Figure 4. 14). Mean RH was rarely above 90%, rather it was found to be around 60-80% (Figure 4.14).

The main rainfall events occurred on the 3rd December 2007, with 35.6 mm recorded, and on the 26th March 2008, with 24 mm recorded (Figure 4.15). All other rainfall

events were below 20 mm. The mean January temperature for the season was 18.2°C and the mean growing season temperature from flowering to harvest (December 2007- April 2008) was 16.3°C.

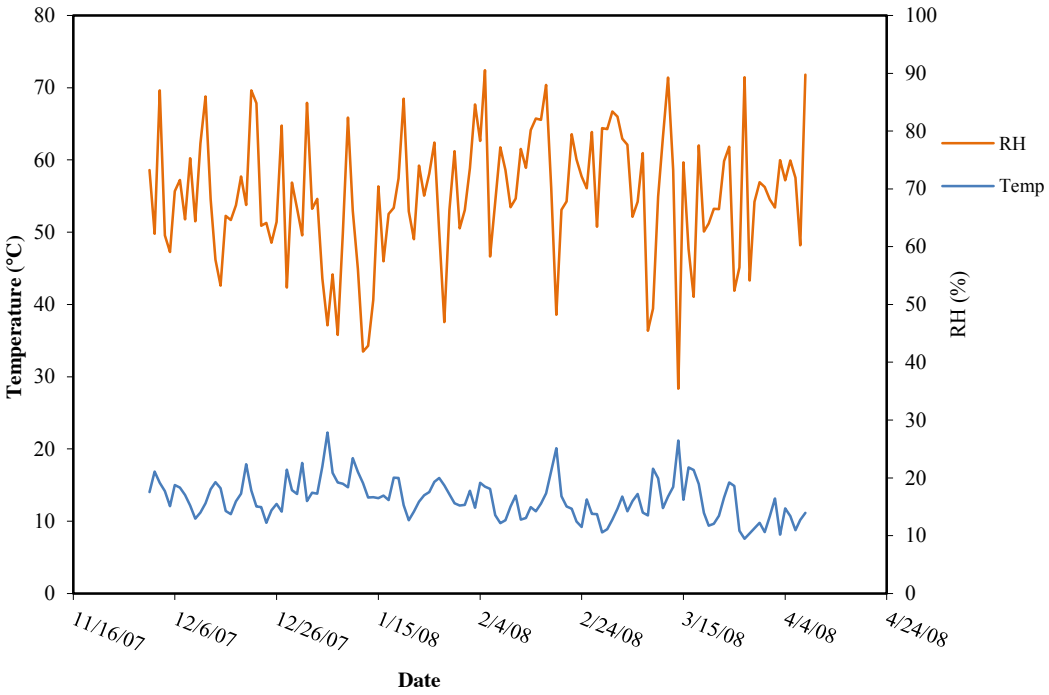

Figure 4.14: Mean daily temperature (Temp, °C) and mean relative humidity (RH, %) recorded during the season by the weather station at the vineyard.

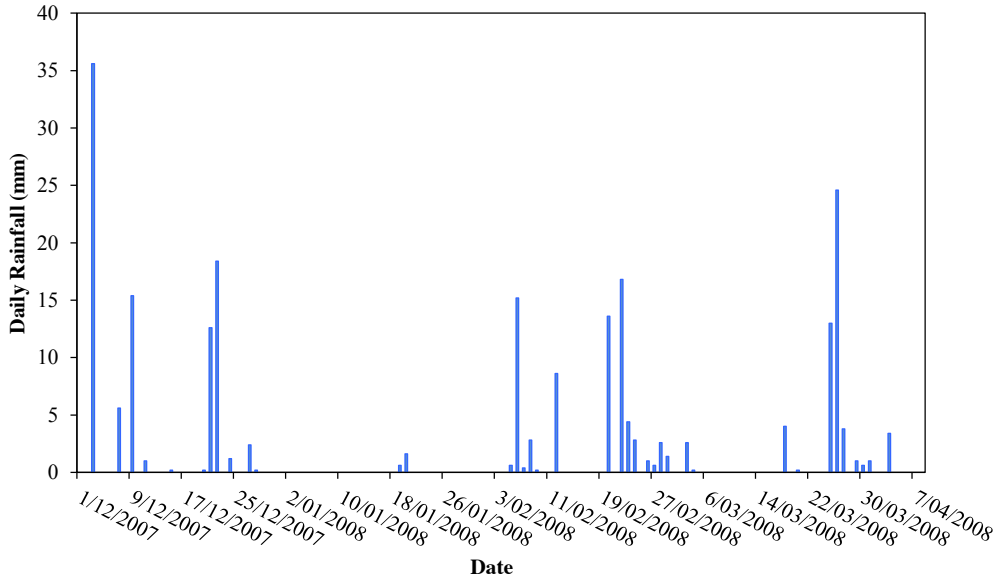

Figure 4.15: Total daily rainfall during the season from 1/12/2007 to 8/04/2008. Data recorded by the weather station at the vineyard.

4.4. Discussion

Results of the field trial conducted during the 2007-08 season indicated that fungicide application had a small effect on BBR severity relative to non-treatment. However, the climatic data collected showed that the season was not highly conducive to BBR development, resulting in a maximum severity close to 5%. The collection of juice samples enabled three methods, qPCR, ELISA and MIR, to be examined to measure the amount of *B. cinerea* present. ELISA and MIR could be used to discriminate fungicide treatments or categories of BBR severity to some degree. To the author's knowledge, qPCR had not previously been applied to juice samples for quantification of *B. cinerea* DNA. Even though the application of the technique appeared to be sound, qPCR or the corresponding visual disease assessment did not result in significant differences among treatments, unlike the results for ELISA. This results suggests that the qPCR method needs further development in order to produce more robust results in order to be able to be applied in this manner.

The investigations conducted in this trial, relating to removal of flowering/ canopy trash, found that it was not a significant source of inoculum for BBR development. The incubation of the bunch trash collected showed very low levels of colonisation by *B. cinerea*. These results and the poor correlation with visual BBR severity suggest that the necrotic tissue pathway was not the main infection pathway at this trial site. The results obtained in this study support the previous findings of Rozario *et al*. (2005) in which trash removal did not significantly reduce the amount of BBR observed when applied in conjunction with the fungicide treatments. For necrotic bunch trash to be a significant source of inoculum, it has been noted that moist weather conditions are needed in order for the fungus to sporulate after the initial colonisation (Rozario *et al*. 2005; Warren *et al*. 2005; Jaspers *et al*. 2013). The weather data collected during the trial showed that the season was relatively dry with limited free moisture available to promote colonisation and inoculum build up. Jaspers *et al*. (2013) also noted that there was variation between vineyards with regard to the level of colonisation of plant matter by *B. cinerea*, as well as the types of plant matter (aborted, aborted berries, calyptras and stamens) colonised by the fungus

within bunches. With this in mind, further investigation is needed using a number of different vineyard sites and grape varieties over several years to determine the role of bunch trash as a source of inoculum in Tasmania. It could also be useful to investigate sampling at later stages during berry development than that of this study, due to the low incidence of *B. cinerea* detected in the collected bunch trash.

Isolation of *B. cinerea* at the grapevine growth stages of pre-bunch closure (PBC) and *véraison* in the incubated green (unripe) fruit strongly suggests that the fungus became established in the fruit between flowering and early berry development. This observation and those for bunch trash incubation suggest that the latent infection pathway was the main pathway for this vineyard site. Keller *et al*. (2003), Nair *et al*. (1995), Pezet *et al*. (2003) and Holz (2003) have all previously shown that the fungus can become established during the early phases of grape fruit development.

The mid-season sprays at both grapevine growth stages of pea-size and PBC reduced the incidence of latent *B. cinerea* in berries at both sample dates (PBC and *véraison*) compared with the nil fungicide treatments. These results suggest that these stages in a crop protection program could provide effective control in minimising latent infection establishment, therefore potentially reducing end-of-season severity. Studies by Beresford and Hill (2008) have found that when there was a high incidence of latent *B. cinerea*, disease severity observed at the end of season was also high. However, their study also highlighted that the use of latent infection, incidence was not a reliable method for predicting minor epidemics and that there was a large amount of variation in the data collected. Once latent infections are established, it is thought that fungal growth resumes during ripening and breaks down the berry tissue resulting in BBR symptoms (Keller *et al*. 2003; Pezet *et al*. 2003). However, relying on observations of latent infection may not predict the end of season severity or incidence, and subsequently fruit quality (de Kock & Holz 1994; Dubos & Roudet 2000).

The purpose of applying fungicides is to prevent or minimise the outbreak of the disease in a crop. Several studies into BBR management have demonstrated that a more targeted approach to spray application can achieve disease control equivalent to that of a full season spray program (Mundy and Beresford 2002; Agnew *et al*. 2004b;

Edwards *et al*. 2009). This small plot trial supports these reports that a reduced number of targeted spray inputs can reduce BBR severity, since there was a trend in which the mid-season applications reduced the severity and incidence of disease compared to that of the nil treatments. The trifloxystrobin treatment resulted in similar incidence levels to that of the PBC cyprodinil + fludioxonil treatment; however, the reason for this observation is obscure. There was a marked increase in BBR severity between the 2nd of April assessment and the 7th of April assessment, which coincided with a rainfall event. Prior to this, BBR severity was quite low, and remained steady. The post-harvest incubation results indicated significant differences and showed that the trifloxystrobin-treated bunches resulted in less sporulation than the control and other fungicide treatments, but had a severity greater than the pea and PBC fungicide treatments. Given the relatively low BBR incidence and severity observed, the results from the experimental use of trifloxystrobin suggest that it needs to be further explored over a number of seasons to fully determine its efficacy in minimising *B*. *cinerea* sporulation under high disease pressure. It should also be noted that the fungicide treatments in this study were not compared with a full fungicide regime, and cannot be compared directly with the results of other trials using full fungicide regimes (Mundy and Beresford 2002; Agnew *et al*. 2004b; Evans *et al*. 2010a). This trial highlighted the importance of the mid-season sprays coinciding with berry development, which reduced and delayed both severity and incidence of the disease as the season progressed.

Harvest disease severity is the variable of most interest to industry; however, other disease variables and analyses can sometimes give more insight on how treatments affect disease development over time. While time was a significant factor in the progression of BBR severity and incidence, AUPDC and repeated measures analyses failed to provide additional information on treatment effects. Nevertheless, the linear progression model generated predictive dates at least 7 days after the actual harvest date for each of the treatments to reach the 5% severity threshold. The results highlight the importance of weather conditions for BBR development. Previously conducted research in other regions over a number of seasons has noted that weather plays an integral part in the amount of *B*. *cinerea* that is expressed (Thomas *et al*. 1988; Emmett *et al*. 2005; Warren *et al*. 2005; Wilcox *et al*. 2006; Beresford 2007). Although there were a number of rainfall events during the present study, they were

all followed by long periods of dry weather. In the two weeks prior to harvest, there were several rainfall events close together, resulting in a rapid increase in severity and incidence of BBR. The results from predictive curves generated also suggested that the amount of sporulation of the fungus observed in the field played a key role in the progression of BBR, which is highly weather dependant. This would explain the lower severity predicted and the delayed dates at which the threshold would have been reached. It should also be noted that the PBC treatment without trash removal had an earlier predictive date for 5 % BBR severity, which did not correspond with the other mid-season treatments (PBC with trash removal and the pea-size treatments). The raw data for this treatment showed that there were more bunches with severity above 10% than the other PBC treatment. This result might have eventuated from an error in the application of the fungicide by hand-held sprayer leading to sub-optimal spray coverage of the fruit. Vine factors did not appear to explain this result, as vine vigour throughout the trial site was similar (data not presented).

Bunch compactness correlated with BBR severity as the basal bunches, which were significantly more compact than the distal bunches, had higher BBR severity in the nil fungicide treatment than the less compact distal bunches. There have been a number of studies that have shown that compacted or tight bunches are more predisposed to BBR and other bunch rots (Vail *et al*. 1998; Sharvrukov *et al*. 2003; Hed *et al*. 2009). This characteristic is also highlighted where some varieties are more susceptible to BBR, which include Chardonnay, Sauvignon blanc, Riesling and Pinot noir (Nair and Parker 1985; Vail *et al*. 1998; Elmer and Michailides 2004; Howell 2011). All of these varieties have a tendency to produce tight bunches, where the epidermal and hyperdermal layer could become compromised, enabling the fungus to infiltrate berries (Gabler *et al*. 2003). Tight bunches would also apply increased pressure on berries, forcing them to burst, providing a wound site and a source of nutrients for the fungus to grow.

Botrytis cinerea DNA was successfully extracted from and detected in juice from naturally infected grape samples. However, the qPCR method was not able to determine significant differences in *B*. *cinerea* DNA between field treatments due to the variation that was observed. It should also be noted that the analysis of the visual

BBR severity scores of the bunches from which the juice originated showed no significant differences between treatments. The high variation observed in the qPCR results may be due to sample amount, which was a sub sample of the original juice collected, with only one subsample per juice sample taken. A larger sample size may have proved to be of benefit and potentially reduced the variation that was observed. Another aspect, which may account for the variation is the DNA extraction process. The amount of DNA extracted may vary between samples and there is also the risk of PCR inhibitors being retained after all the cleaning steps.

The qPCR method was able to detect *B. cinerea* in all juice samples, highlighting the utility of using juice samples rather than berry samples, with the sample preparation being quicker and more versatile, as well as being more representative of what may be present within a bunch. The method shows the potential of using the qPCR technique to detect and track the amount of *B. cinerea* present in grapes during the season at any growth stage. The DNA extraction method, that was further adapted from the work previously described by Lin & Walker (1997) and Cadle-Davidson (2008), showed that DNA could be successfully extracted for the purpose of detecting *B. cinerea* in grape juice samples. Previous studies have shown that using grape juice or wine as a source for DNA samples to determine fungal and bacterial contaminates has been successful (Delaherche *et al*. 2004; Gindreau *et al*. 2006; Stummer et al 2006). To date, the qPCR method has only been commercially adopted for measuring and detecting Brett taint caused by bacteria. However the qPCR method is gradually becoming widely used for research purposes (Culbert *et al*. 2008). Further development in the future may allow it to be adopted in the commercial environment, as at this stage it is a tool more suited to research, as the results have shown here.

The QuickStix™ test was the only method that significantly distinguished between treatments, as opposed to the qPCR and MIR methods, and the observed BBR severity using ANOVA. The test proved to be a quick and simple way to measure the amount of *B. cinerea* present in the juice as a SI score (signal intensity) in grapes. There was no significant clustering with regards to SI category for the PCA analysis. The Tukey-pairwise analysis showed that the low SI readings correlated with lower BBR severity resulting in the group being significantly different to the high SI group that had greater mean BBR severity. However the medium SI category was not

significantly different to the high SI and low SI groups. The assay works on a basis of recognition antibody recognising the corresponding antigen in the sample, which can result in error, and or limited capabilities of quantification. For quantification purposes it requires a special reader, as used in this study.

Preliminary investigations into the use of the QuickStix™ test in berries harvested at the PBC growth stage found that it would not accurately quantify *B. cinerea* in the sample (data not presented). It is at this stage when *B. cinerea* is thought to be in a latent phase with no active growth. Celik *et al*. (2009) found that the ELISA based method only worked well when *B. cinerea* was actively growing in grapes. This finding supports the results that were obtained for the PBC berries in this study. Overall it could be said that the use of ELISA is only suited for use during the ripening phase of the berry, when the *B. cinerea* fungus is actively colonising the berry, resulting in the visual symptoms. It is a test that was designed to be easily used at the weighbridge to determine fungal contaminates levels in the harvested fruit, nor for the detection or measuring of latent infections established in the developing berry prior to the ripening phase (Dewey *et al*. 2005).

Results of the application of MIR spectroscopy indicated that it only weakly correlated with the BBR severity scored visually. The lack of correlation with the other two methods (qPCR and QuickStix™) may be because they are each measuring a different component, which may or may not correlate. These are: 1) MIR determines the wavelengths in the juice sample, 2) qPCR measures the quantity of *B. cinerea* DNA in the juice sample and 3) the QuickStix™ measures the quantity of *B. cinerea* antigens present. BBR causes oxidation in the juice as it secretes enzymes to break down the tissue, thus affecting fruit and wine quality (Bulit and Dubos 1988; Godden 2000; Dumeau *et al*. 2004). This might impact on the MIR readings, as it measures reflectance of the molecules present in the juice sample. This would also explain why a weak correlation was found with the observed BBR severity, as the score is the amount of symptomatic tissue present in the bunch, in which the chemical changes would be taking place as the berry rots. To date there has only been limited published investigations into the use of spectroscopy for measuring BBR (Cozzolino *et al*. 2003; Versari *et al*. 2008). Where spectroscopy has been used successfully, the studies have noted that it correlates with the compounds such as gluconic acid and

glycerol, which appear in the chemical makeup of the berry/ juice due to BBR infection (Versari *et al*. 2008). As this was a preliminary experiment to complement the qPCR and ELISA work, the results suggest that further investigation using MIR or NIR in determining BBR levels is needed, before any method similar to that for the use of colour analysis is adopted by industry (AWRI 2009).

The qPCR method is suited to research rather than industry application due to the technical expertise required for the method to work, including modifications to suit equipment and reagents used. Each of the methods explored in this study was described in Chapter One Section 1.9. This study confirmed that the ELISA tool was simple to use and resulted in statistical differences between the fungicide treatments. Mid infrared (MIR) spectroscopy was the easiest method to apply because it required very little sample preparation; however, the results were not correlated to those of ELISA or qPCR. The ELISA method is unlikely to be applied in the vineyard as an alternative to visual scoring, but it may prove to be a useful option at the winery to fine-tune remedial wine making, or as a research tool to quantify BBR incidence and severity when levels are too low to obtain statistical separation of treatments by visual scoring. Further research and development are needed for IR spectroscopy, which presumably would require some form of on-the-go sensing to provide a practical in-vineyard assessment. It would also require a calibration method, such as an improved assay for qPCR. This technique, once developed, might be taken up faster in the winery as it uses spectroscopy equipment that is already an essential piece of equipment in winery laboratories. Potentially it would require fewer reagents than either qPCR or the ELISA method.

4.5. Conclusion

Overall, the study found that although fungicide timing had a significant effect on BBR incidence, it had less effect on BBR severity. The key driver of BBR severity was rainfall close to harvest. Both mid-season treatments at pea-size berries and PBC appeared to reduce BBR severity at harvest, with moist incubations of bunches at harvest showing significantly less *B. cinerea* colonisation of the berries relative to non-treatment. Time was also a significant factor with regards to the development of the disease. Trifloxystrobin was found to reduce sporulation and had some impact on

overall BBR severity, warranting further detailed investigation. The necrotic tissue pathway was found to not be a significant driver in BBR development, with the limited isolation of the fungus from the necrotic flowering trash examined. This study also demonstrated the usefulness of novel approaches for detecting and measuring the amount of *B. cinerea* in grape samples, complementing or even potentially replacing visual observations. The QuickStix™ test was found to distinguish treatments, while the visual data resulted in less conclusive results. Application of the qPCR method was successful in the detection of *B. cinerea* DNA in grape juice, highlighting the potential for the method if treatment differences exist. DNA extraction from grape juice samples was shown to be successful and less time consuming than from berries, with potential applications in the tracking of *B. cinerea* throughout the season, using the modified DNA extraction process. Results from the use of this method warrant further investigation and development of the technique as a research tool. Preliminary investigations into the use of MIR to determine BBR levels in juice found that it correlated with visual scoring and not the other two quantitative methods for the samples tested.

Chapter Five
Whole of block study on botrytis bunch rot

5.1. Introduction

Botrytis cinerea, the cause of botrytis bunch rot (BBR), can add significant costs to the production of fruit and wine in Australia and worldwide (Godden 2000; Scholefield and Morison 2010; Lorrain *et al*. 2012). The fungus can become established during flowering and the initial stages of fruit development, after which it goes into a latent phase until fruit ripening when symptoms appear (Elmer and Michailides 2004). Crop and environmental variability from one growing season to the next influences the temporal progression (location in time and/or rate) of any plant disease epidemic.

In BBR management, spray timing can play an integral role in minimising disease expression at harvest (Edwards *et al*. 2009). The key spray timings for botrytis at high-risk sites are flowering, pre bunch closure (PBC) and *véraison* (Mertely *et al*. 2002; Wicks 2002; Agnew *et al*. 2004; Emmett *et al*. 2005; Braybrook 2007; Edwards *et al*. 2009). However, it is not always appropriate to spray at all these stages due to cost, industry regulations and weather. Recent harvest events (2007-08 and 2010-11 season) have demonstrated that even applying a full regime of fungicides will not necessarily protect the crop from severe BBR, as weather conditions influence the outcome of disease development (Agnew *et al*. 2004; Riley 2008; Edwards *et al*. 2009). During a season when conditions are not favourable for BBR development there is opportunity for growers to reduce the amount of fungicides they apply (Agnew *et al*. 2004; Edwards *et al*. 2009). Best practice disease management includes a combination of vine management (cultural methods), spray application and timing (Gubler *et al*. 1987; Wolf *et al*. 1997; Balasubramaniam *et al*. 2000; Kingston 2001; Mertely *et al*. 2002; Mundy and Beresford 2002; Wicks and Hall 2003; Agnew *et al*. 2004; Cole 2004; Cole *et al*. 2004; Cole 2005). Studies have been conducted

into the effect of vine vigour (Valdés-Goméz *et al*. 2008), bunch characteristics (Vail *et al*. 1998; Dry and Thomas 2003; Hed *et al*. 2009) and soil moisture (Wilcox *et al*. 2006) on BBR severity and incidence. However, the studies were conducted under either controlled environments (e.g. potted vines) or small-plot trials without consideration of what may occur in a commercial vineyard environment. To date there has been little investigation into the role of other factors contributing to BBR development across whole vineyard blocks.

There are many factors that contribute to the spatial variation in any given farming scenario, affecting both product quality and disease severity. In all vineyards, there will be some variation across the site. This can include variation in soil type, which may impact drainage and water logging, nutrient availability and plant health. There will also be variation in vine vigour. All of these factors can have a direct or indirect impact on fruit and wine quality (Bramley and Williams 2001; Dobrowski *et al*. 2003; Bramley and Hamilton 2004; Lanyon and Bramley 2004; Bramley *et al*. 2005a; Bramley 2005; Bramley 2007; Acevedo-Opazo *et al*. 2008; Bramley 2010a; Bramley and Trought 2010; Hall *et al*. 2011; Panten and Bramley 2011). Spatial variation is often not considered when studying plant disease epidemics and the effects of treatments within the target crop, as trials are usually conducted using replicated small plots (Jacometti *et al*. 2007; Edwards *et al*. 2009; Evans *et al*. 2010b; Reglinski *et al*. 2010). By using the whole-of-block experiment, under commercial settings, the understanding of spatial variation within a site and over time is achieved; whereas in smaller field trials or controlled experimental sites this is unachievable (Bramley *et al*. 2005b; Bishop and Lark 2006; Bramley 2007; Panten *et al*. 2010).

Precision Viticulture (PV) was developed based on the principles of Precision Agriculture (PA) in order to account for spatial variability across a vineyard, using specialised equipment to generate spatial maps (Bramley and Hamilton 2004). The design of most field trials in viticulture is the 'small' plot trial, with each plot being a panel of vines, ranging from one vine to six vines (depending on vine spacing) per treatment. The design of these trials may not take into consideration the full extent of spatial variation that occurs across a site as they are generally set up in one section within a vineyard block (Bramley and Lanyon 2003; Bramley 2010a; Panten and Bramley 2011). Precision Viticulture is a tool, which can be applied in both research

and practical vineyard management and can lead to a greater understanding of the target crop and the environmental factors that affect fruit yield and quality at harvest (Proffit *et al*. 2006).

The PA approach has been used in a number of trials studying fungal infections in wheat crops, using the whole-of-block layout (Jacobi and Kühbauch 2005; Larsolle and Hamid Muhammed 2005; Tartachnyk *et al*. 2005). Whole-of-block experimentation can be described as a field trial; as the name suggests, where the site incorporates the whole block in which the host crop is used for the study, rather than a small section of the block as regularly used in randomized plot trials (Bramley 2007; Panten *et al*. 2010). Remote sensing techniques have been used to study grapevine downy mildew caused by *Plasmopara viticola* (Stoll *et al*. 2008), and to track and predict phylloxera infections (Bruce *et al*. 2009). However there has been limited application of the whole-of-block method of experimentation in the area of grapevine diseases. This method has been applied in a study of powdery mildew caused by the fungal pathogen *Erysiphe necator* (Bramley *et al*. 2007, 2011), which investigated alternatives to synthetic fungicides in managing the disease using organic practices.

The purpose of this study was to investigate the epidemiology of BBR and to gain a further understanding of factors that contribute to BBR incidence and severity using the 'whole of block' experimental trial method. The role of spray timing in a commercial vineyard was examined to build on previous small plot investigations in previous seasons and those reported by Edwards *et al*. (2009) and Evans *et al*. (2010b). Variation in vine vigour, fruit characteristics, soil moisture and clone were investigated for their role in contributing to BBR development. The quantitative PCR (qPCR) technique developed in Chapter Two was used to test its suitability for detecting natural infections of *B. cinerea* in grape berries.

5.2. Methods

5.2.1. Trial site & layout

The trial site was located within a commercially managed vineyard situated in the Rokeby region of southern Tasmania, east of Hobart. An east-facing 2.4 ha block of *V. vinifera* cultivar Chardonnay with an elevation range of approximately 23 m was used. The block consisted of three clones as follows from north to south: I10V1 (Tasmania: 8127) (rows 1-23), Penfolds (rows 24-43) and G9V7 (Tasmania: 2306) (rows 44-60), all planted in 1998 (Figure 5.1). The trellis system was Scott Henry with vine and row spacing of 1.35 m and 2.4 m, respectively, with an east-west row orientation. The row orientation was different to that generally accepted for Tasmanian vineyards, where orientation is generally north south to allow for airflow (wind) down the row and even sun exposure. Airflow was across the rows at this site. The vines were subjected to leaf removal at about PBC (EL31) and hedged prior to the application of bird nets at *véraison* (EL34) as per normal vineyard practice. The soil profile varied from loamy clay soil at the top of the block to a silty clay loam at the bottom. Determination of the grapevine growth stages for the trial was based on the modified EL system of Coombe (1995).

The trial consisted of two treatments: fungicide application at flowering or at pre-bunch closure. There were 10 blocks each with six rows, with each treatment applied to alternate blocks to produce five replicate blocks (Figure 5.1). The fungicide used for both treatments was Switch® (Syngenta Group, Basel, Switzerland, 375 g/kg cyprodinil plus 250 g/kg fludioxinil) and was applied at 80g/100 L with 0.01% non-ionic surfactant (Agral® Syngenta Group, Basel, Switzerland). The vineyard personnel were responsible for all mixing and application of fungicide mixtures. The fungicide was applied using an air shear sprayer (Silivan Turbo Sprayer) (Figure 5.2), which was powered by a Fendt 280P tractor with the output rate of 780 L/ha. The first application was completed at 80% capfall (applied on 15th December, 2008) and will be referred to as 'flowering'. The second treatment was the application of the fungicide at pre-bunch closure (PBC), applied on 27th January 2009 (referred to as 'PBC'). The vineyard was also sprayed for other diseases and pests as per vineyard

manager's management regime with the exclusion of multi-target fungicides that might have had some activity against *B. cinerea*.

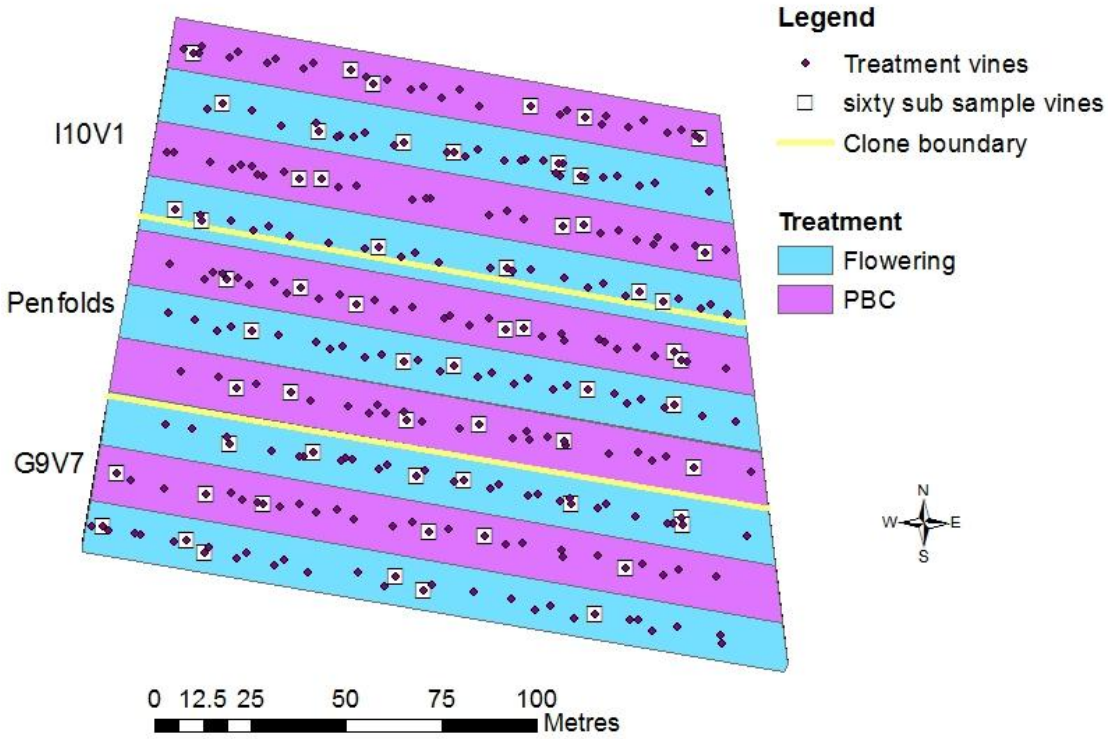

Figure 5. 1: Map showing the treatment layout (flowering and PBC) of the trial site with the 300 tagged vines that were used to collect the data. Also shown is the 60 sub sample of vines, which were used to collect yield data. The yellow lines show the boundary of each of the 3 Chardonnay clones in the block (I10V1, Penfolds and GV7).

Figure 5. 2: Block in the midst of fungicide application by the grower co-operator.

Each treatment consisted of 150 vines that were used for collection of disease and vine data (refer to Figure 5.3 for position of tagged vines in the block). The middle two rows of the six-row block were used to select the vines to be studied and to ensure negligible spray deposit from an adjacent treatment. Initially every 6th vine was selected per sample row, with 30 vines per replicate (each shaded area in Figure 5.2). Using random number generation in GenStat®, 15% of the selected vines were deselected, and another vine selected either adjacent to or diagonally opposite from a randomly selected vine (Bramley 2005). An Excel® spread sheet was used to assist in positioning sample vines (one vine per worksheet cell) prior to them being geo-referenced using a differentially corrected global position system (dGPS), which is accurate to approximately 50 cm in the *x* and *y* planes. The block boundary was also geo-referenced.

5.2.2. Determination of B. cinerea incidence using the overnight freezing incidence test (ONFIT)

Fifty bunches (twenty-five basal and twenty-five distal) from each treatment were harvested at PBC twenty-four hours after the PBC spray. The method used to determine the incidence of *B. cinerea* in green berries was that described by Evans *et al*. (2010b), which is based on an original method developed by Lou and Michailides (2001) (refer to Chapter Four for detailed description of the method). Bunches were randomly harvested from the two middle rows of each block and placed into polythene bags, which were then placed in the freezer at –18°C for at least 24 h. The ONFIT technique involves moist incubating a 20-berry subsample from each bunch, post freezing and surface disinfestation, at room temperature (15 - 20°C) with exposure to daylight via windows. The subsamples were assessed with the aid of a stereomicroscope after 6 and 11 days of incubation. The presence of *B. cinerea* on a detached berry was confirmed if the characteristic conidia and conidiophores were observed. The incidence of *B. cinerea* was the percentage of berries showing signs of

B. cinerea. The incidence of other fungi (e.g. *Penicillium*, *Aspergillus* and *Rhizopus* spp.) was also noted when assessing the berries for *B. cinerea* incidence.

5.2.3. Disease assessments

For each vine, a total of 12 bunches (6 basal and 6 distal) were tagged for the visual assessments. The visual assessments were performed on the 10th, 18th and 24th of March, and on 2nd of April 2009. Two days were required to complete each assessment, except for the final assessment, which was performed over 3 days due to adverse weather conditions that occurred in the middle of assessment on the second day. At each assessment, bunches were scored for percentage area of the bunch with symptoms of BBR, which was broken down into pink brown turgid berries (new infections), shrivelled pink brown berries, pink split berries, as well as total BBR. The percentage of berries with sporulating *B. cinerea* was also identified. For this chapter, results from total BBR are shown, whereas the different categories of symptoms are reported in Appendix F. Bunches were also assessed for other diseases present in the fruit, percentage of splitting and other damage (insect/ bird damage, mechanical) and on the final assessment, sunburn damage. The disease severity scale that was used in the assessments was that used in previous field trials (scale = 0% - 100%)(Appendix D) (R.W. Emmett, Department of Primary Industries, Victoria, personal communication).

5.2.4. Soil moisture and conductivity

During the initial dGPS survey of the block boundary and the target vines, a high-resolution electromagnetic induction (EM38) survey was completed by Mr Neil Meadows (Terrapix, Lenah Valley, Tasmania). This EM38 equipment measures the bulk soil electrical conductivity. This is a measure of the amount of clay, salts, minerals and water in the soil, spatially mapped to show the variation in the soil properties across the block (Proffit *et al*. 2006). The survey was completed on the 11th February 2009. To obtain measures of soil moisture during the season, nine G-bugs (gypsum blocks) (GB lites) (MEA, Adelaide) were placed throughout the block in a grid pattern (Figure 5.3 & 5.4). Mr Justin Direen of the Tasmanian Institute of

Agriculture placed each sensor in the ground at 400 mm depth on The 6th of January 2009. Refer to Figure 5.6 for the position of the installed G bugs in the trial block). An 800 mm length of 10 mm-diameter PVC pipe was attached containing the cords to which the reader could be attached, to the end of the G-bug and sealed with PVC glue. Prior to installation in the ground, the sensors were left to soak in water for 10 min and the hole to which the sensors would be placed filled with water. The sensors attached to the PVC pipe were then placed in the hole ensuring all air was expelled. The hole was then backfilled around the PVC pipe with fine soil that had been augured out. The position was recorded using GPS (Oregon 300, Garmin, USA). A handheld reader was used to obtain the soil moisture tension readings (kPa). This is a measure of how tightly water is bound to soil particles, thus how difficult it is for the plant to extract the water (MEA, Adelaide). The lower the kPa value, the wetter the soil resulting in greater amount of plant available water. The data obtained were used to determine if soil moisture was associated with the severity of bunch rot. Readings were taken regularly during the season and after rainfall events (next day).

Figure 5.3: Gypsum block installed in the ground with the associated reader used to take soil moisture readings.

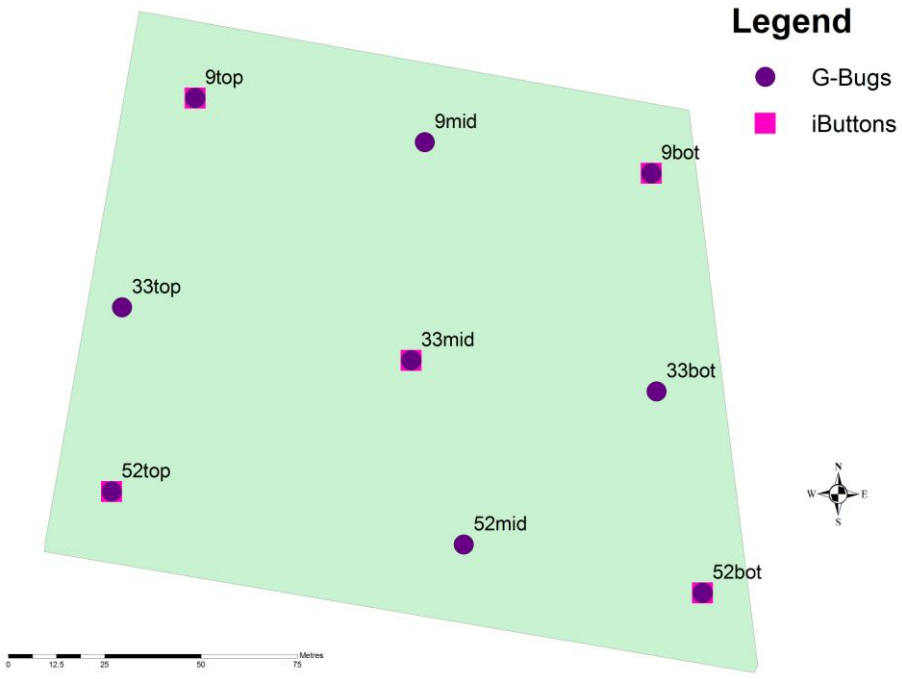

Figure 5. 4: Figure of the block showing the position of the G-Bugs (GB lites) that were installed at the trial site. Also shown is the position of the five environmental monitoring stations that collected temperature and humidity readings in each of the canopies.

5.2.5. Determination of vine vigour

Vine vigour was assessed throughout the season using several techniques. During ripening (EL 33-35), the Point Quadrant technique (Smart and Robinson 1991) was used to determine canopy density, which is a measure of the amount of vegetative growth of the vines. Two rows were selected from each of the three clones, with rows randomly selected. Within each row, panels were counted and from the western side, panels 3, 8, 13, 18 and 23 selected. The assessment was completed on the 25th of February 2009. The method involved inserting a thin metal rod into the fruit zone of the canopy and counting the number of leaves, bunches and clusters touching the rod. Insertions were placed at 10 cm intervals for each of the panels selected for a total of 10 insertions per panel. The number of gaps, interior and exterior leaves and bunches were recorded. The gap percentage, interior bunches, interior leaves, leaf layer

number and bunch exposure was calculated as described by Smart and Robinson (1991).

For measurement of harvest yield and pruning weights, a subsample of 60 vines (30 per treatment) from the 300 in total was used. The vines were selected by using every fifth vine for each of the treatments (refer to Figure 5.3 for the vines sub sampled). Harvest measures collected included total crop yield (kg) and bunch number. For pruning data, vines were manually cane pruned as per normal vineyard practice. The number of canes removed was recorded along with cane length and weight (kg). The number of canes remaining after pruning was also recorded along with cordon length to give a mean kg pruning weight/meter as regularly used in industry practices, to gain an understanding of overall vine vigour, balance and fruit production.

Trunk diameter has been used to assess variation of vine vigour across a block in a number of trials (Cortell *et al*. 2005; Bennett *et al*. 2007; Trought *et al*. 2008). It is a measure of cumulative vigour of the vine, which is a measure of its vigour over its life span to date. Trunk diameter measurements were taken over three days in the first week of May 2009. The method used was based on the published method by Trought *et al*. (2008) which involved taking the diameter of the trunk at 20 cm above soil and 15 cm below the head of the vine and calculating the average between the two measurements.

Smart Viticulture organised aerial imaging of the block to obtain a high-resolution (50 cm) image of the plant cell density index (PCD) at *véraison* during the following growing season (2010). PCD was calculated by using the ratio of reflected light at near infrared and red wavelengths, where the difference corresponds to the amount of vegetative growth. In many published articles relating to both agricultural and viticultural research, PCD has been found to be a useful measure of plant vigour (Dobrowski *et al*. 2003). The survey was unable to be obtained during the actual trial season due to the unavailability of the service; however, results of studies conducted by Acevedo-Opazo *et al*. (2008), Bramley and Hamilton (2004) and Bramley *et al*. (2011b) suggest that there is little spatial variation in grapevine vigour among seasons for the trial sites studied.

5.2.6. Juice Characteristics

Tracking of ripeness during the season

Juice assessments were performed weekly after commencement of *véraison* and involved measuring ^{o}Brix (total soluble solids). Six out of the 60 rows were used for sampling for Brix measurements. The same rows and panels were used as that for the point quadrant vigour assessment, allowing for 3 rows per treatment to track the ripening. Each sample consisted of 10 randomly picked berries sampled across the selected panel, with a total of 50 berries per row.

Harvest samples

The 60 vines used for yield assessments (refer to Section 5.2.5 for sample selection) were also used for juice characterisation; however, only bunches tagged for disease assessment were placed in a bag for juice analysis. Brix was measured using a refractometer (Pocket PAL-1, Atago, Japan); pH and titratable acidity were measured using an auto-titrator (Metrohm Instruments, Switzerland).

5.2.7. Application of qPCR to quantify B. cinerea

Berries were sampled for qPCR assays 2 days before commercial harvest on 6th April, 2009 (1 day prior the harvest of the 60 tagged vines). Sampling involved arbitrarily selecting 50 berries from 50 bunches on the vines sampled for disease assessment and the two vines on either side of the sample vine. Where two tagged vines were situated side-by-side, two untagged vines either to the eastern or western side of the vines were also used. The 50 berries were pooled for DNA extraction.

The technique for DNA extraction was described in Chapter Two, section 2.2.2. DNA concentration was quantified (section 2.2.3). Samples for which concentration was above 5 ng/µL were normalised to this value using sterile Mill Q water. Samples for which concentration was below 5 ng/µL were tested using the qPCR assay and not normalised. Previous experiments in this study (Chapters Two and Three) during the

initial qPCR method development and application found that where total DNA was not able to be quantified after the extraction process, the qPCR assay did detect either or both *B. cinerea* DNA and *V. vinifera* DNA. This resulted in some qPCR reactions containing less DNA per reaction than the samples that used normalised DNA samples were used. Each qPCR reaction contained 12.5 µL 2× StrataGene Brilliant® II qPCR Master Mix (StrataGene, Agilent Technologies, California, USA), 0.3 µM of each primer (either or both KJD BcF and BcR or KJD GF and GR), 0.2 µM of probe (KJD BcP and or KJD GP), 2.5 µL of DNA sample with the volume made up to 25 µL. The cycling conditions were as follows:- 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 50°C for 1 min and the extra extension step of 72°C for 15 s, all of which were perform in a RotorGene 3000 real-time qPCR machine (formerly Corbett Life Sciences (Qiagen)). Each run contained four no-template controls (water), 4 negative controls (undiluted *V. vinifera* DNA) and one positive control (undiluted *B. cinerea* DNA). Samples were quantified using the dilution series as described in Chapter Two (refer to Table 2.2 and Figure 2.6 for amounts). Each batch of qPCR assays comprised 25 different samples tested in duplicate. A total of 16 test batches were completed over 9 days. A sample was assayed again if the first assay failed to detect either *B. cinerea* DNA or *V. vinifera* DNA in a sample, or the result appeared to be an outlier. The assay failed if DNA of either organism was not detected after the assay was repeated.

5.2.8. Environmental data

Five ibutton sensors (Thermochron, Alfa-Tek Australia Pty Ltd) were placed throughout the block to determine if there were differences among them in temperature and humidity. Row number increased from north to south. Initially the environmental sensors were placed in rows 4 and 46 (elevated western side), 34 (middle) and 15 and 58 (low lying/ eastern side). On the 6th January, the sensors were moved to the same position as the soil moisture probes. At the bottom (eastern side) and top of the block (western side), the sensors were placed in rows 9 and 52 and the middle sensor was placed in row 33 (Figure 5.4 for position in the trial block). Sensors were placed in the fruiting zone directly above the soil moisture probes. The sensors were attached to a clear plastic cover to protect from damage during spraying

and to aid attachment to the trellis wire using zip ties (Figure 5.5). Data collection for each sensor commenced on the 12th November 2008 and was recorded hourly until harvest. The sensor placed in row 4 (elevated/ western side) failed to collect relative humidity readings from the 12th November to 1st December 2008, and sensor 52 (top) malfunctioned between 17th February and 12th March 2009.

A separate weather station consisting of Tinytag data loggers (Gemini Data Loggers (UK) Ltd) was also erected on the eastern side of the block (Figure 5.6). The data loggers were located 1.6 m above the ground as recommended by Beresford and Spink (1992). Average air temperature, relative humidity, surface moisture and rainfall were recorded at 10 min intervals. The data logger for temperature and relative humidity (Tinytag Ultra 2 (dual channel)) was housed in a plastic shelter (Hastings Data Loggers (HDL), Port Macquarie, Australia). A tipping bucket rain gauge (Rain Collector II, Davis Instruments, USA) was used to collect rainfall data and was calibrated to tip every 0.2 mm of rainfall. The leaf wetness sensor (Model 237, Campbell Scientific Inc. Utah, USA) was mounted at a 10° angle in order to minimise surface moisture run-off and the cable was modified to enable connection to a Tinytag data logger. The readings for the main weather station commenced on the 7th November 2008 and finished on the 4th April 2009.

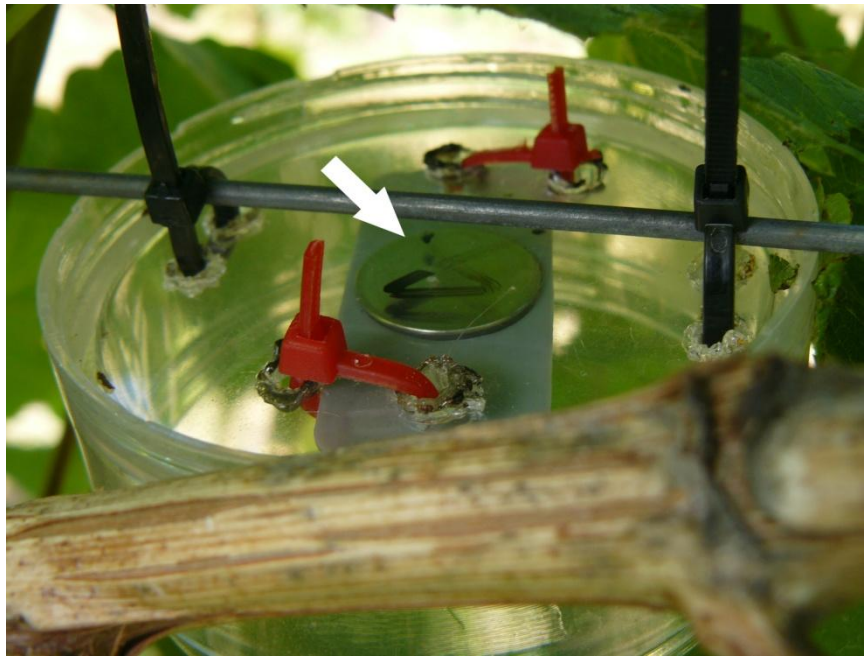

Figure 5.5: Installation of the ibutton to record temperature and humidity within the canopy during the season. White arrow in figure is pointing at the iButton.

Figure 5.6: Weather station installed at trial site.

5.2.9. Data analysis

All data collation and summation was performed using Microsoft Office Excel (2008) unless specified. For the analysis of temporal data, the dates on which the data were collected were transformed to a number using the Microsoft dating system starting at January 1, 1900. All disease severity data was logit transformed as described by Beresford *et al*. (2006) unless specified (refer to equation B6 Appendix B). All statistical analyses except that of spatial mapping was completed using the statistical software package GenStat® 10th edition version 10.1 (VSN International Ltd, UK). Simple one sided *t* test analysis was used to determine treatment differences using the logit-transformed severity of BBR, sporulation and pink plump berries at harvest. This method was also used to analyse data generated from the qPCR analysis, ONFIT and temperature differences between the western and eastern sides of the block. Linear regression analysis was completed for the visual assessment scores for BBR severity using the logit transformation described previously. Regression analysis was also used to determine if there was a correlation between BBR severity and total vine pruning weight. This method was also used to analyse the standard dilution series used in the qPCR assay. Analysis of Variance (ANOVA) was used to determine if there were significant relationships between the spray treatment (BBR severity) and vine factors, which included vigour (according to PCR imagery grouping), clone and bunch position using the transformed BBR severity. This method was also used to determine if bunch position was found to be a significant factor for the ONFIT results. ANOVA was also used to determine if there were significant differences between the four ibuttons in the block and the main weather station for temperature for each month. Standard error (SE) and least significant difference (lsd) values were also calculated for all data analysed. Unbalanced ANOVA was used when n values were not the same to determine if there were significant interaction between treatment and vine characteristics.

5.2.9.1. Analysis of qPCR data

The qPCR data was collected using the RotorGene software supplied with the real-time PCR machine; analysis of raw data was done using a later version of the software RotorGene Q, Pure Detection (version 1.7, Build 94) (Qiagen Pty Ltd). Reaction efficiencies were calculated using the equation described by Bustin *et al*. (2009) (Equation B2, Appendix B).

Percentage *B*. *cinerea* DNA was calculated for each sample by dividing the amount of *B*. *cinerea* DNA measured by the total amount of DNA (ng) used in each reaction. Overall treatment means were also calculated. For each treatment, samples were divided into those that had ≥ 350 fg of *B*. *cinerea* DNA and those that had < 350 fg *B*. *cinerea* DNA to create a 2 x 2 contingency table. The limit of 350 fg was chosen as it was the standard with the lowest concentration of *B*. *cinerea* DNA. Chi-square analysis was completed to identify treatment differences, excluding samples where the assay failed.

When treatment means were compared, percentage *B*. *cinerea* DNA was arcsine transformed using the equation B10 mentioned in Appendix B prior to *t* test analysis. A non-parametric Mann-Whitney U test was also conducted to determine if there were treatment differences using both the transformed percentage *B*. *cinerea* DNA and untransformed values due to the data not being normally distributed, according to the analysis using GenStat® 10th edition. Unbalanced ANOVA was completed to determine if there was any interaction between clone or vine vigour and fungicide treatment for the amount of *B*. *cinerea* DNA detected.

5.2.9.2. Geostatistical analysis and mapping

The geostatistical analysis and mapping was performed in collaboration with Dr Rob Bramley (Principle Research Scientist, Precision Agriculture, CSIRO Ecosystems Sciences, Adelaide, SA). The geostatistical methods used were those proposed by Bishop and Lark (2006) for the analysis of spatial variation at the landscape scale. The method assumed that the treatment response was a spatially auto-correlated and cross-correlated random variable. It resulted in the estimation of treatment response for any section of the trial site using the response data for all treatments. The response variables (disease severity) were tested for conformity to a normal distribution by testing for skewness and using octile skew as a measure of asymmetry. When the calculated skewness was outside the range of -1 and 1 and -0.2 and 0.2 for testing of octile skew, the data was transformed by log or square root transformation (Brys *et al*. 2004; Lark *et al*. 2006).

Data from the flowering treatment were used to generate temporal spatial maps due to the observed higher severity and greater variation than that of the PBC treatment. The transformed data derived from the total BBR severity for this treatment was interpolated using ordinary global kriging and a common global variogram derived for each date (Webster

and Oliver 2007). The global variogram was produced by offsetting the data from each date by 1000 m in an east-west direction allowing for data to be spatially modelled simultaneously and to ensure the differences between the maps produced were independent of artefacts of the variogram fitting process on any of the dates (Lanyon and Bramley 2004). Maps generated in this study using the transformed data were produced using ArcMap (v 9.3 and 10; ESRI, Redlands).

The data generated from measuring the trunk diameter of the 300 vines was used to generate a spatial map on total vine vigour. Mean trunk circumference data were used to interpolate a map using VESPER software (Minasny *et al*. 2005), after which the krigged data were then further analysed and a final variogram generated using ArcGIS (v9.3 and 10).

5.2.9.3. Temporal analysis for BBR

Temporal curves were developed using treatment means for total BBR severity at different times during berry ripening. Using the maps generated for the final assessment, zones were derived for each of the treatments to determine the different epidemics that were occurring in the block over time. Means were generated from each of the zones, via pooling of the vines that were identified in each zone. To determine the temporal progression of BBR severity according to vine vigour, the PCD image was used to separate the vines into groups according to vine vigour and separate curves generated for each vigour category and fungicide treatment. The area under the disease progression curve (AUDPC) was calculated for each vine in each zone and used to determine statistical differences in disease development according to the different regions of the block. This method is widely used in the temporal study of plant disease development (Jeger and Viljanen-Rollinson 2001; Mohapatra *et al*. 2008). Repeated measures of analysis was also used as a tool in conjunction with the AUDPC analysis to determine if there were significant differences between treatments, epidemics and interactions with treatment with vigour and clone over time. The data used in this analysis was the severity and incidence data obtained through the visual scoring of BBR at each of the four time points.

5.3. Results:

5.3.1. ONFIT (Moist Incubation)

Very little *B. cinerea* was observed 6 days after incubation of immature berries. After 11 days of incubation, the mean incidence of *B. cinerea* in the berries taken from the flowering treatment (3.0%) was approximately twice that of the PBC treatment (1.4%), but these differences were not statistically different at P<0.05 (one sided t-test using log transformed mean incidence) (Table 5.1). Further analysis via ANOVA found that bunch position was not a significant factor either. *Penicillium* and *Aspergillus* species were also found at very low incidence in both treatments (Table 5.1).

Table 5.1: Mean percentage incidence (%) of latent *B. cinerea*, *Penicillium* and *Aspergillus* infections in berry samples taken at PBC from both the flowering and PBC treatments after 11 days of incubation. Standard error of the means (SE) is shown in brackets.

	Pathogen Assessed		
	B. cinerea (SE)	*Penicillium* (SE)	*Aspergillus* (SE)
Flowering	1.4 (0.85)	0.7 (0.32)	0.2 (0.20)
PBC	3.0 (1.10)	0.5 (0.26)	0.3 (0.33)

5.3.2. Disease Severity

The pre-bunch closure spray was more effective ($P < 0.001$ for one sided t test) in controlling BBR than the flowering spray (Table 5.2), with total BBR severity at harvest (3rd April) being 1.5% and 3.5% respectively. There was a slight decrease in BBR severity between the 24th March assessment and the final assessment on 3rd of April 2009, possibly due to some diseased berries dropping to the ground.

Statistical analysis of the logit transformed data comparing the two treatments found that on both the 25th of March 2009 and the 3rd April 2009 (harvest) the flowering treatment resulted in significantly higher incidence of BBR (one-sided *t* test, $P < 0.001$), severity of BBR (pink brown turgid berries and total BBR severity (shrivelled + turgid)), and sporulation ($P< 0.001$)(refer to Figure 5.9 for sporulating *B. cinerea)*.

A high amount of what appeared to be coulure and millerandage disorders commonly referred as 'hen and chicken' (Mullins *et al*. 1992) were observed in the grape bunches (Figure 5.9). It was observed that there was a high amount of splitting (Figure 5.7) in the smaller sized berries (Figure 5.8). The splitting also resulted in a higher amount of bunch rots other than BBR being observed in bunches where the berries had not dried up completely (refer to Appendix F, Table F1). The other rots found in the block included sour rot, *Penicillium* and *Aspergillus*. Some split berries also showed signs of *B. cinerea* infection.

Ripening varied across the block, which may have also influenced disease severity. Prior to harvest, bunches were scored for percentage sunburn damage, with berries a golden brown colour rather than the normal green translucent colour. Sunburn damage ranged from as little as 5% to 95% of the bunch. The majority of the sunburn damage was towards the middle of the block, rather than towards the western side.

Table 5.2: Mean total BBR severity (%) across the four assessment dates during ripening of *Vitis vinifera* cv. Chardonnay for the fungicide programs 'flowering' or 'PBC' for the 2008-09 season. Percentage *B. cinerea* sporulation and BBR incidence (number of bunches with symptoms) are also shown. Standard error (SE) is shown in brackets. Refer to Table F1 in appendix F for data for the different BBR categories.

Date	Flowering			PBC		
	Severity	Sporulation	Incidence	Severity	Sporulation	Incidence
10th March	0.19 (0.03)	0.06 (0.01)	175	0.40 (0.05)	0.09 (0.01)	263
18th March	0.29 (0.05)	0.16 (0.03)	179	0.35 (0.04)	0.16 (0.02)	218
24th March	3.62 (0.32)	2.74 (0.29)	816	1.64(0.15)	1.03 (0.12)	568
3rd April	3.49 (0.31)	2.73 (0.30)	780	1.51 (0.17)	0.99 (0.15)	507

Figure 5.7: Example of berry splitting with examples of both old infected shrivelled split berries and relatively fresh splitting. Photo taken at harvest.

Figure 5.8: Example of a bunch showing uneven berry set and variation in berry development, taken around EL 32.

Figure 5.9: Botrytis infected bunch at harvest showing sporulation by *B. cinerea*.

Spatial variation in BBR severity between treatments

BBR severity was spatially variable for both treatments in the pre-harvest period, increasing towards the western (upslope) side (Figure 5.10). BBR severity decreased between 24th March and the 3rd April. Spatial variation in the level of statistical significance (*P* value) between treatments varied with date, with a greater proportion of the block presenting *P* values < 0.05 for the later (April) disease assessment (Figure 5.10). The significance of difference maps in Figure 5.10 show that the minimum severity level of the flowering treatment for a statistical difference between treatments was 3.61% (1.9 % square root transformed, (Figure 5.10)), while the PBC treatment maximum severity was 1.3% (1.69% square root transformed, (Figure 5.10)). These thresholds were found to decrease slightly for the 3rd of April, where the minimum severity for the flowering and PBC treatments to 2.25% (1.5 % in Figure 5.10) and 1.21- 1.5% (1.1 % in Figure 5.11) respectively was required for a statistical significance. Overall the maps show that the PBC treatment was more effective than the flowering treatment.

5.3.3 Temporal change in the spatial distribution of BBR severity

The spatial maps generated using data from the flowering treatment allowed comparison of maps for each assessment date (Figure 5.11). Disease was not only more severe on the western, upslope side, it also appeared that the increase in severity appeared to be more rapid on this side of the block, with BBR severity increasing rapidly between 18th March and 24th March (Figure 5.11). The group of maps situated on the right-hand side of Figure 5.11, show the same data except that they are grouped in 20th percentiles and show that the patterns of BBR severity variation is similar for the block for each date, except that there is a trend for increasing severity. These patterns were also apparent in the common variograms generated using the the *k* means for each date to generate the map layers into zones of similar severity. Overall this figure suggests that the pattern of disease was that the severity increased at the initial site over time and was not due to the spread of BBR to new infection sites in the block (Figure 5.11).

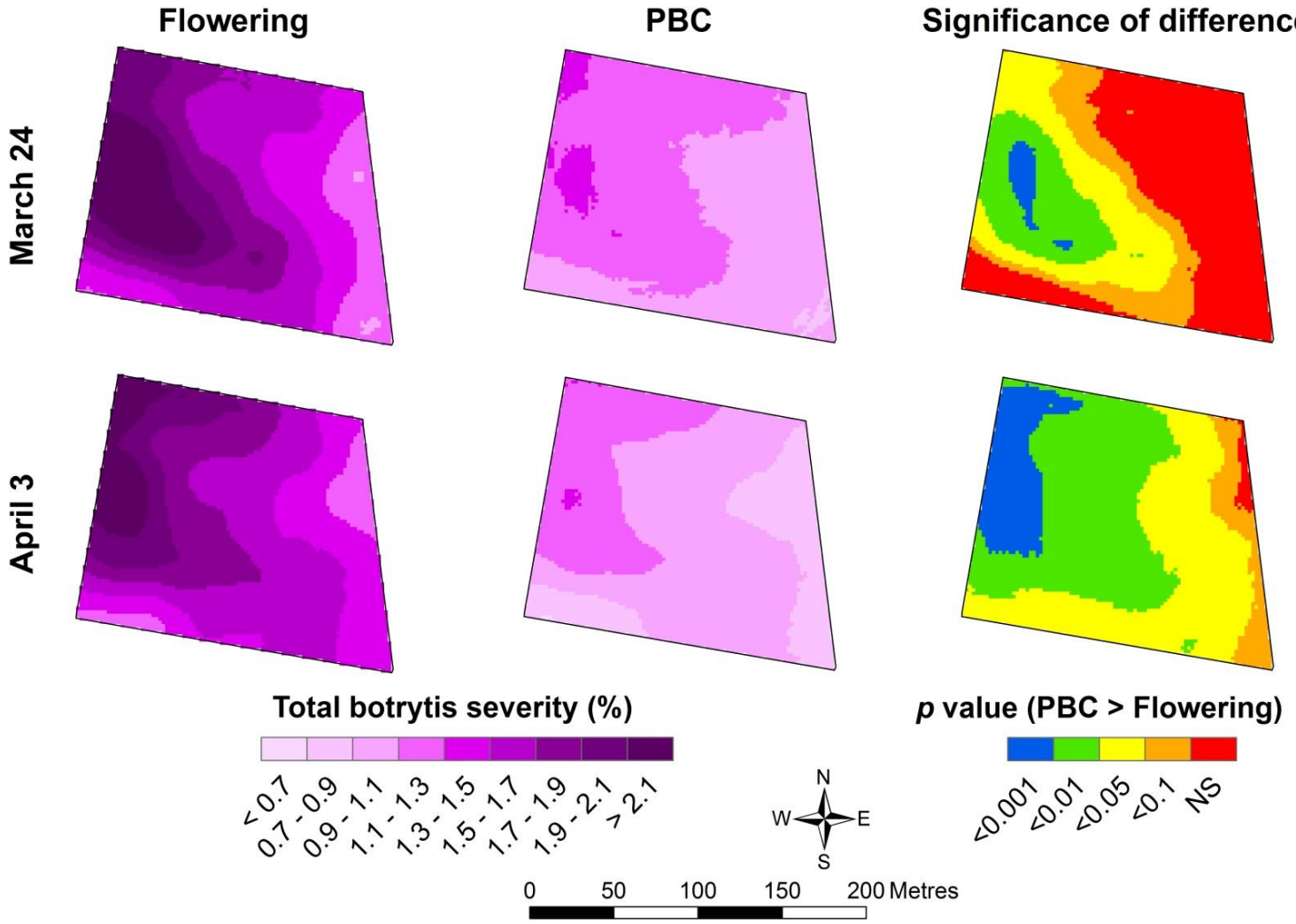

Figure 5.10: Spatial variation in the severity of botrytis (square root transformed data) across the trial site for each of the fungicide treatments flowering and pre-bunch closure (PBC). Maps were generated by Dr Rob Bramley (CSIRO) using data from immediately prior to harvest (April 3) and approximately 10 days earlier (24th March 2008).

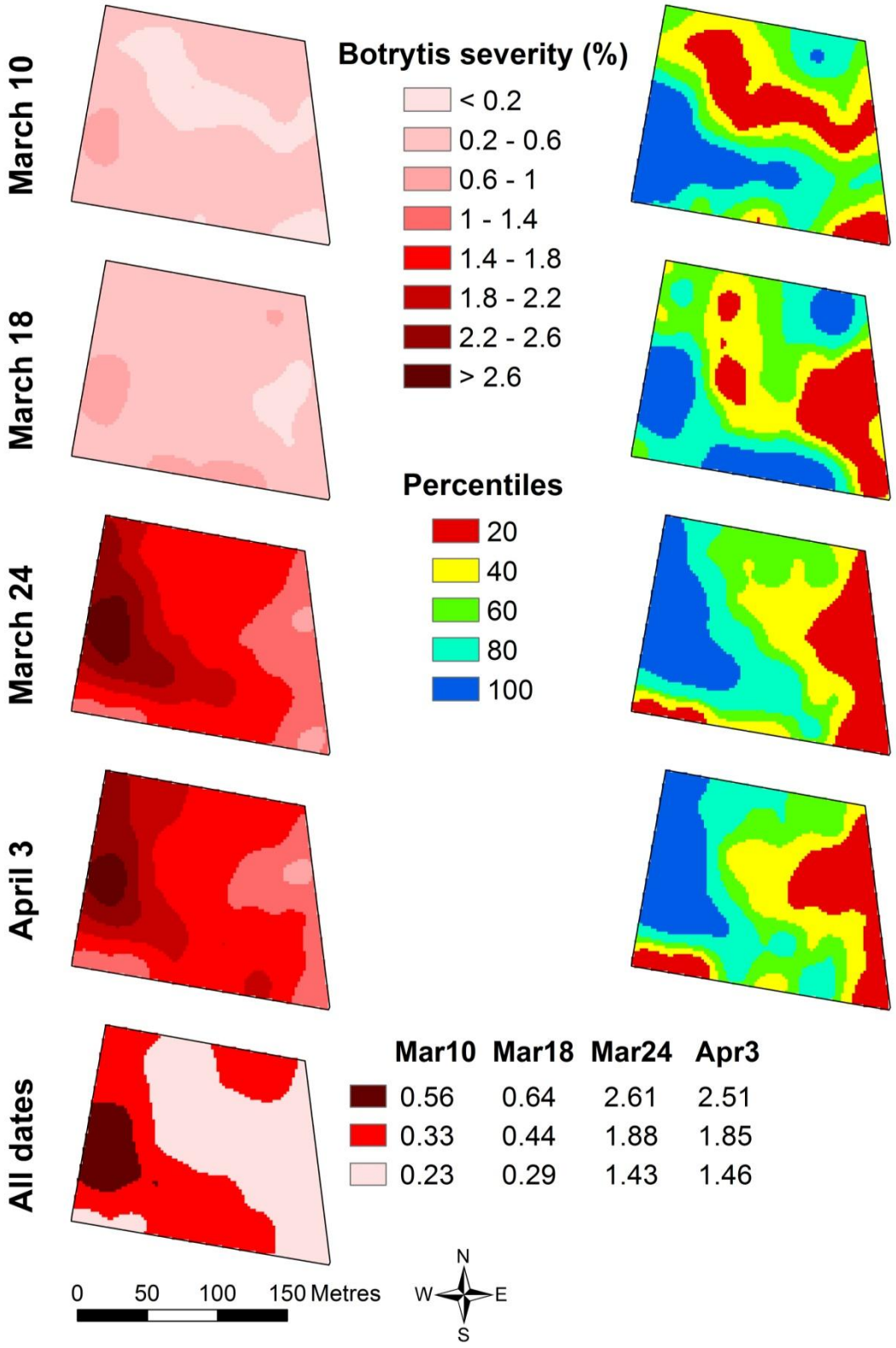

Figure 5.11: Temporal change in the spatial distribution of botrytis severity (%) using the flowering treatment vines. Data was square-root transformed prior to mapping and interpolated using a common variogram (all dates map). The two panels show the same data except that they are either classified using equal intervals (left panel) or 20th percentiles (right panel). Maps were generated by Dr R. Bramley, CSIRO.

5.3.4. Temporal progress curves of BBR severity

5.3.4.1. Flowering

For the flowering treatment, the spatial variogram derived from the BBR severity data at harvest was used to divide the block into five severity groups (F1 (blue), F2 (violet), F3 purple), F4 (magenta) and F5 (orange); Figure 5.12). Analysis of Variance of the areas under the disease progression curves (AUDPC calculated per sample vine) showed that section F1 had a significantly higher mean AUDPC than the other sections (Table 5.3), with higher severity observed at the last two assessment dates (Figures 5.12 and 5.13). Section F5 had the least AUDPC (Table 5.3). Sections F1 and F2 were shown to have mean higher BBR severity than that of the overall treatment mean, showing that there was more than one BBR epidemic occuring within the block. Analysis of each temporal curve found that the regressions were not significant (P= >0.05; Figure 5.13) for each section; therefore, predictive curves were not generated.

Table 5.3: Mean AUPDC for each epidemic (F1-F5) associated with the flowering treatment (refer to Figure 5.13 for different epidemic regions). The number of vines for each epidemic is shown. ANOVA was conducted using each vine's calculated AUDPC. Means for the area under the disease progression curve (AUDPC) sharing the same letter are not significantly different at *P* = 0.05.

Block Section	AUDPC (SE)	n vines
F1	105.88 (22.02) a	20
F2	58.48 (6.86) b	33
F3	41.20 (3.79) bc	56
F4	29.15 (5.16) bc	25
F5	18.80 (6.09) c	16
lsd	25.41	-
P Value	< 0.001	-
Residual df	145	-
Flowering Mean	*49.23*	*150*

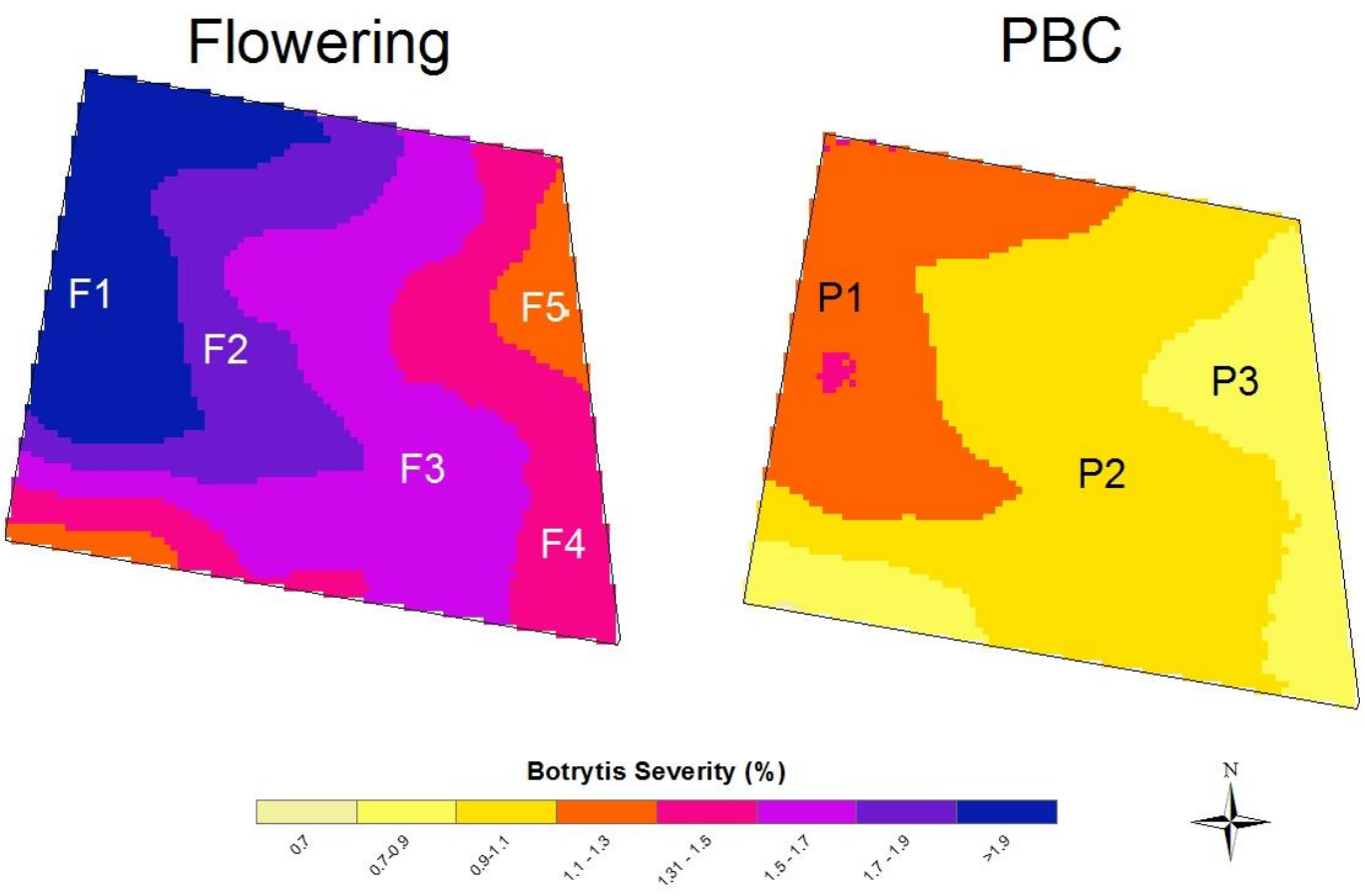

Figure 5.12: Zones of trial block according to severity at harvest for both treatments used for developing temporal curves for botrytis severity (%). The flowering treatment was divided into five sections (F1-F5) and the PBC treatment a total of three sections (P1-P3). Spatial maps were derived based on the square-root transformation of the data.

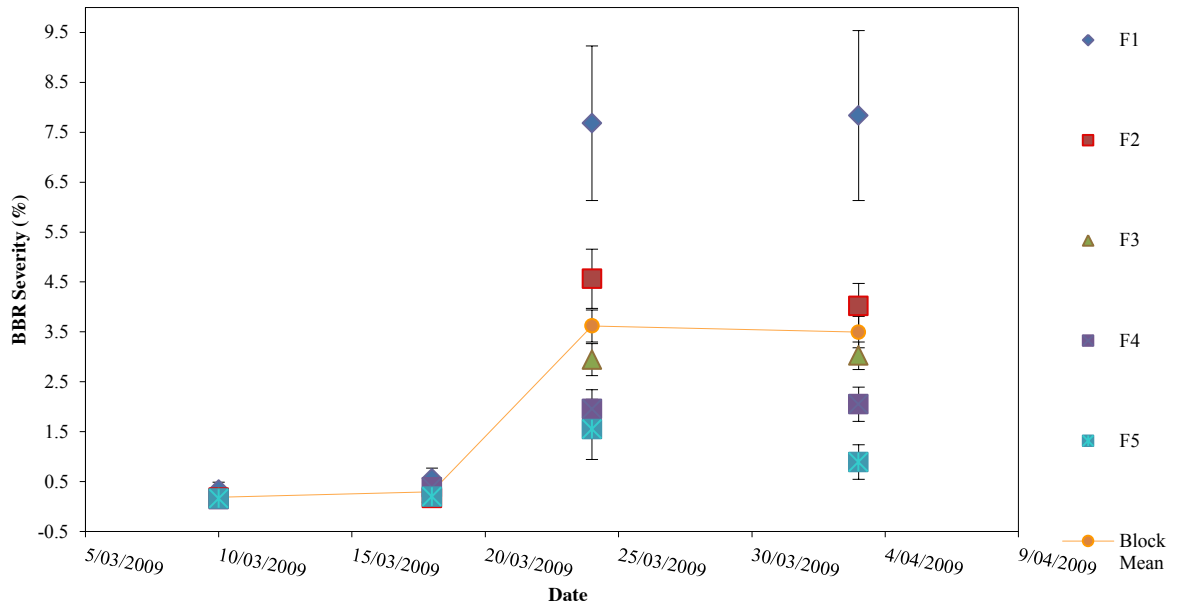

Figure 5.13: Temporal progression of total BBR severity (%) for the different sections of the block as represented in Figure 5.13 for the flowering treatment. Error bars show standard error for BBR severity at each assessment date.

5.3.4.2. PBC

Disease severity within the block could be divided into three sections (P1 (orange), P2 (dark yellow) and P3 (yellow)) for the PBC treatment (Figure 5.12), which were used to investigate variation in the temporal progression of BBR. Section P1 had the highest severity at the final two assessments dates (Figure 5.14) and was situated towards the top and west (Figure 5.12) of the block. Section P2, situated towards the middle of the block, followed a similar pattern to P1 although the severity was less than the block mean. In the P3 section, the increase in BBR severity over time was less than the P1 and P2 sections and the overall treatment mean. Additionally, the increase was steady across the dates and not a significant jump at the last two assessment dates (Figure 5.14). The ANOVA results using AUPDC data showed that there was a significant difference between P1 and P3 epidemics, with P2 not being significantly different from the other two epidemics (Table 5.4). As the linear regressions (Figure 5.14) were not significant (P> 0.05) predictive curves were not generated.

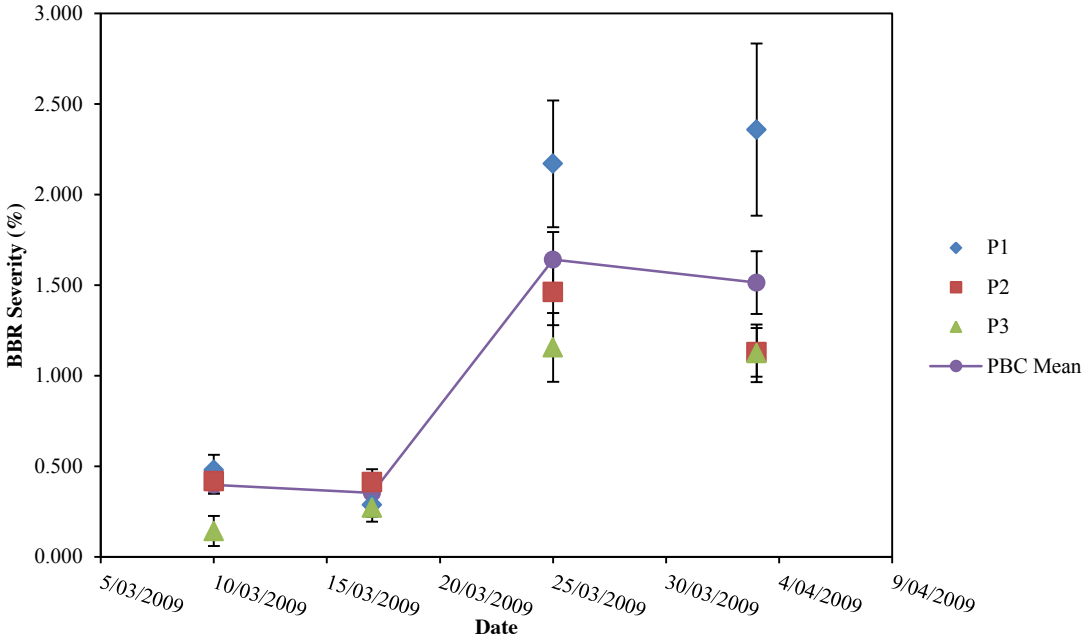

Figure 5.14: Temporal progression curves for BBR severity for sections of the block as displayed in Figure 5.12 (PBC map). The severities for each section were P1 (1.1 - 1.3); P2 (0.9 - 1.1) and P3 (0.7 - 0.9). The mean severity of the PBC treatment is shown as a purple line.

Table 5.4: Mean AUPDC for each epidemic (P1-P3) associated with the PBC treatment (refer to figure 5.13 for different epidemic regions). The number of vines for each epidemic is shown. ANOVA was conducted using each vine's calculated AUDPC. Means for the area under the disease progression curve (AUDPC) sharing the same letter are not significantly different at *P* = 0.05.

Block Section	AUDPC (SE)	no vines
P1	33.08 (5.34) a	47
P2	21.90 (2.29) ab	82
P3	17.34 (2.57) b	21
lsd	*11.82*	-
P Value	0.026	-
df	147	-
PBC Mean	*24.764 (2.16)*	*150*

[a] Number of Assessments during the season.

5.3.5. Incidence of BBR over time

The repeated measures analysis showed that there was a significant interaction between treatment and time according to BBR incidence (Table 5.5). As for the BBR severity results, the flowering treatment resulted in greater BBR incidence than the PBC treatment.

Table 5.5: Repeated Measures ANOVA of BBR incidence over time with fungicide treatment and time as factors.

Source	df	SS	MS	F value	P > F
Fungicide Timing	1	9113.4	9113.4	13.33	< 0.001
Residual	298	203810.9	683.9	3.78	-
Time	3	197898.0	65966.0	364.11	< 0.001
Time. Fungicide Timing	3	24892.7	8297.6	45.80	< 0.001
Residual	894	161967.4	181.2	-	-
Total	1199	597682.4	-	-	-

5.3.6 Soil profile & elevation

The EM38 measurements showed that, as expected, the soil conductivity varied across the block (Figure 5.15). According to the vineyard manager, the high conductivity readings in a line along the southern end of the block may have been caused by a row of steel posts. Towards the north-west corner of the block, where the G Bug 9 was placed, the ground was often muddy, which was reflected in the lower kPA values. The lower kPa reading the less effort is required for the uptake of water by the plant, which means that there is more free moisture in the soil (Table 5.6). This was also reflected in the vigour of the vines, as the canopy was relatively dense at the top of the slope. There was no significant correlation between the EM38 map and either BBR severity or the PCD imagery (Figures 5.15 & 5.16, (PCD).

Table 5.6 presents the readings from the G Bugs, showing that available moisture varied during the season and across the block. In general, the top of the block had higher readings, with the G Bug in row 52 giving the highest kPa values. These readings appear to correspond with the PCD image (Figure 5.15 (PCD)), as readily available water resulted in higher vigour vines and increased BBR severity. The middle-of-the-row readings varied across the three rows, with rows 33 and 52 recording higher readings except on the days when the soil was too dry. The bottom of the slope was wetter earlier in the season but drier close to harvest. There was consistently more soil moisture in rows 9 and 33 than in row 52 (Table 5.6 & Figure 5.15).

Table 5.6: Soil moisture readings (kPa) during the growing season measured using gypsum blocks (G-Bugs, GB lites).

Date	Row 9			Row 33			Row 52		
	Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
16/01/2009	108	76	116	36	138	130	180	132	114
10/02/2009	190	60	254	292	326	288	364	358	160
17/02/2009	246	18	284	318	350	232	372	380	224
26/02/2009	24	20	164	334	168	152	350	384	82
12/03/2009	200	13	169	Dry	111	110	Dry	Dry	65
18/03/2009	19	37	Dry	Dry	151	133	179	Dry	24
25/03/2009	57	11	115	Dry	60	123	141	179	50
1/04/2009	14	31	51	Dry	44	127	151	187	20

Figure 5.15 suggests a correlation between the elevation of the block and BBR severity, with both parameters increasing towards the western side of the block. However, the block was surveyed using dGPS and not the more accurate Real Time Kinematic GPS, thus this only a visual interpretation of the maps as a detailed spatial analysis could not be completed.

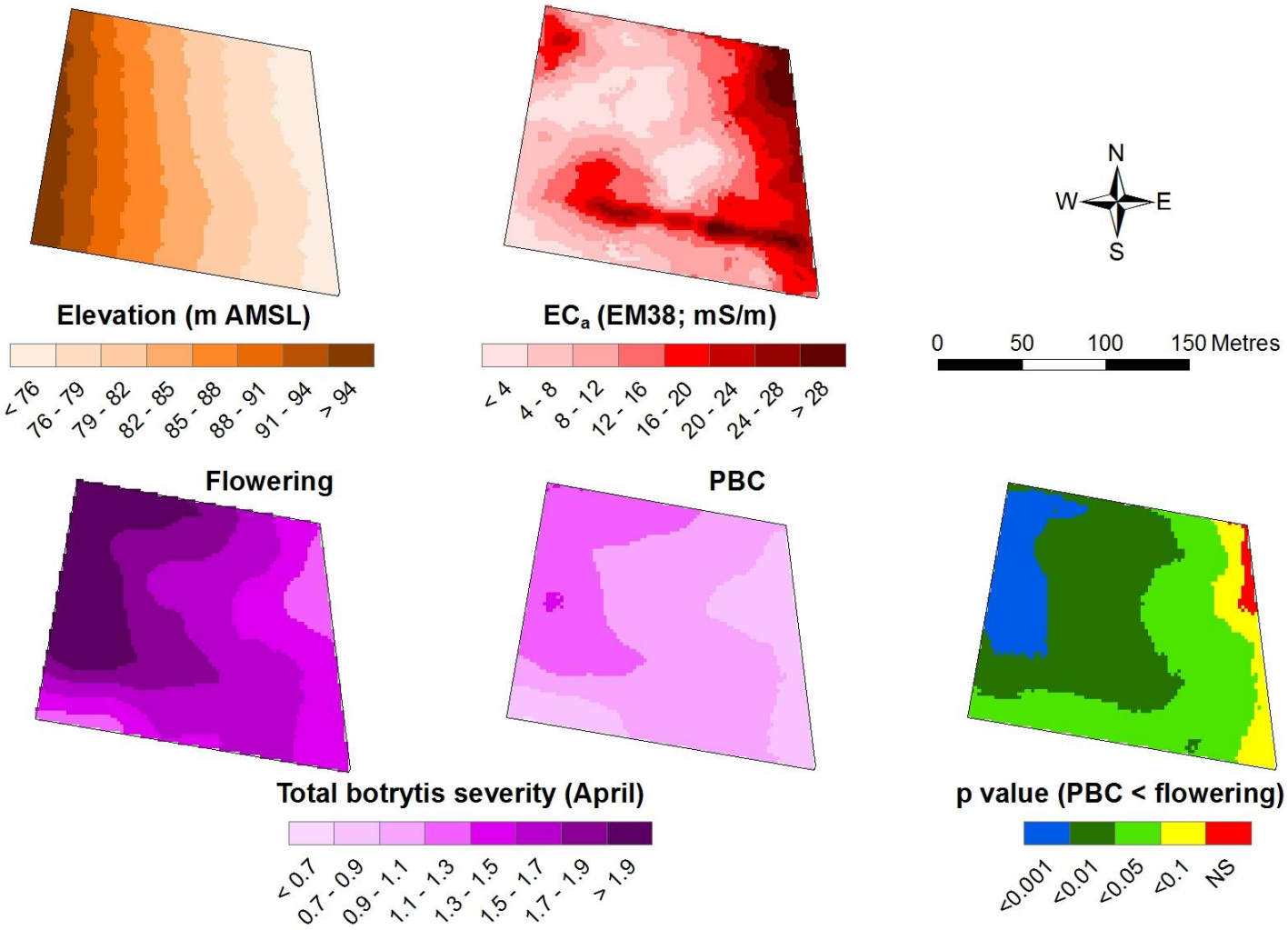

Figure 5.15: Spatial maps showing overall elevation and EM38 (electrical conductivity) readings of the trial block. Also shown are spatial maps derived for each treatment using square root transformation of botrytis severity taken on the 3rd of April prior to harvest, showing that there appeared to be an association between elevation and BBR severity (variogram showing P value- bottom right of figure).

5.3.7. Vine vigour, clone and BBR severity

5.3.7.1. Vigour & clonal characteristics

The PCD imagery showed a significant amount of variation in vine vigour across the block, with vigour highest at the top of the slope, which at ground level canopy was noticeably denser (Figures 5.16, 5.17, 5.18). Trunk diameter measured at the end of the growing season also demonstrated spatial variation across the block, with larger diameter vines at the top of the slope (Figure 5.17). However, it should be noted that trunk diameter is an indication of total vine growth and not a measure of seasonal growth unless prior readings have been taken. The higher vigour zones appeared to have higher disease severity (Figures 5.16, 5.17, 5.19). The *P* values indicating the significances of the differences between the flowering and PBC treatments were lowest in the region with the highest vigour indicated in Figure 5.17.

Trunk diameter was significantly different between the three clones. The Penfolds clone was significantly less vigorous, with smaller mean trunk diameter than that of the other two clones I10V1 and G9V7 (Table 5.7, Figure 5.16). The one way ANOVA of the Point Quadrant assessments showed no significant differences for vine vigour between the three clones or block section (Table F3, Appendix F). However there was a relationship for the percentage gaps according to clone, since the Penfolds clone had the highest percentage of gaps with a mean of 19% and the G9V7 had the lowest (5%) ($P = 0.075$) (Table F3, Appendix F).

Clone G9V7 had lower pruning weights than the other two clones (Table 5.7). Otherwise, the clones did not vary significantly in their yields or ratios of yield to pruning weights. Bunch size and bunch number were lower for Clone G9V7 (Table 5.7). At the top of the slope, the denser canopies resulted in greater vegetative growth (Figure 5.16. 5.17).

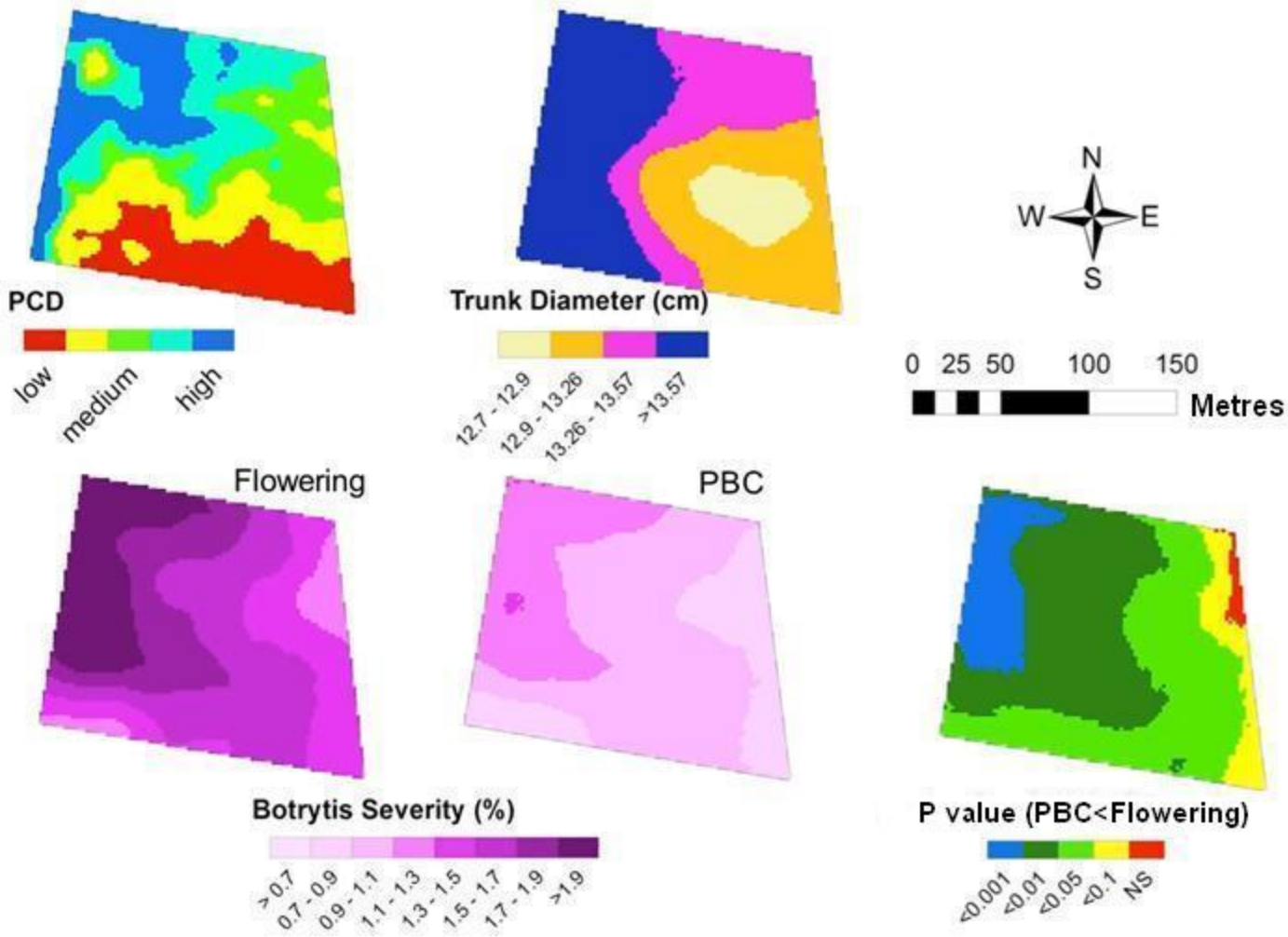

Figure 5.16: Spatial maps of the trial block showing PCD, trunk diameter, and BBR severity for the fungicide treatments and statistical difference between treatments across the block. PCD imagery has shown to be a tool in determining vine vigour, imagery was taken in 2010 one year after trial (Smart Viticulture). Measurements for trunk diameter were taken one month after harvest (May 2009). Trunk diameter maps were derived using VESPER and ArcMap in consultation with Dr Rob Bramley (CSIRO).

Table 5.7: Mean values of yield, trunk diameter and pruning data according to clone. Variation in mean BBR severity according to clone on the final assessment (8th April 2009) assessment. *P* values, Standard Error and LSD are also shown. Within columns, means with the same letter are not significantly different at *P* = 0.05.

Clone	Trunk Diameter (cm)[b]	Yield/vine (kg) [a]	Pruning weight [a]	Yield/Pruning Weight Average [a]	Spray Treatment [b]					
					Flowering BBR Severity (%)	Sporulation (%)	Incidence (%)	PBC BBR Severity (%)	Sporulation (%)	Incidence (%)
I10V1	13.61 (1.16) a	1.34 (0.12)	1.54(0.06) a	0.88 (0.08)	3.44	2.75	51.54	1.69	1.11	51.54
Penfolds	13.09 (1.48) b	1.39 (0.11)	1.40(0.04) a	0.99 (0.07)	4.14	3.49	43.52	1.73	1.13	32.71
G9V7	13.53 (1.50) a	1.09(0.137)	1.26(0.06) b	0.85 (0.09)	3.24	2.34	40.68	0.69	0.43	19.54
P Value	0.017	0.241	0.004	0.450						
LSD	0.381		0.158				-			
df	299	59	59	59						

a Results are based on the 60 sub sampled vines
b Results based on the 300 vines used for the study. Refer to Table 2 in Appendix F for results of factorial ANOVA for BBR severity.

Figure 5.17: Example of the high vigour vines situated toward the top of the block as reflected in the PCD image.

Figure 5.18: Example of the vines, which have lower vigour situated towards the middle and bottom of the block.

Figure 5.19: Example of BBR severity found in the high vigour zone subjected to the flowering treatment.

5.3.7.2. Clone & BBR

There was a significant interaction between Chardonnay clone and spray treatment (Table 5.8). Clones I10V1 and Penfolds had higher BBR severity than Clone G9V7 at final assessment. Repeated measures analysis also showed that there was a significant interaction with fungicide treatment, clone, and time for mean BBR severity over time, but independently it was not significant (Table 5.9).

Table 5.8: Mean BBR severity showing according to spray treatment and clone with results from an unbalanced ANOVA for the final disease assessment (3rd April) ANOVA. ANOVA was completed using logit transformed data (shown in parenthesis), the calculated P values and least significant differences (lsd) are as follows: - clone P = 0.045 (0.3120); spray treatment P = < 0.001 (0.2515); interaction P = 0.013 (0.4398). The lsd is represented in the table via letters next to the mean values, where letters are the same it shows that there is no significant difference between the values.

	Spray treatment		
Clone	Flowering	PBC	Mean
I10V1	3.39 (- 3.71) a	1.40 (-4.64) b	2.40 (-4.19) b
Penfolds	4.08 (- 3.83) a	2.26 (- 4.33) b	3.17 (-3.98) a
G9V7	3.61 (- 3.75) a	0.80 (-5.25) c	2.21 (- 4.46) b
Mean	3.67 (- 3.76) a	1.48 (- 4.73) b	

Table 5.9: Repeated measures analysis of variance for the interaction between the two fungicide treatments (PBC & flowering) and clone for the temporal progression of BBR severity. Analysis based on using the logit-transformed values.

Source	df	SS	MS	F value	P > F
Clone	2	2.4262	1.2131	.54	0.585
Fungicide Timing	1	27.2697	27.2697	12.09	<0.001
Clone. Fungicide Timing	2	21.1525	10.5762	4.69	0.010
Residual	294	663.2995	2.2561	414	-
Time	3	983.1768	327.7256	600.89	<0.001
Time.Clone	6	9.33768	1.5563	2.85	0.015
Time.Fungicide Treatment	3	102.9126	34.3040	62.90	<0.001
Time.Clone.Fungicide Timing	6	10.4797	1.7466	3.20	0.008
Residual	882	481.0452	0.5454	-	-
Total	1199	2301.0993	-	-	-

5.3.7.3 Effect of Vine Vigour on BBR severity

Regression analysis using the BBR severity and pruning weights for the vines in the flowering treatment found that pruning weight accounted for 17% of the variance in logit BBR severity (P = 0.022, R^2 = 0.1739) (Figure 5.20). Analysis of the data using the vines subjected to the PBC found no correlation, this was presumably due to the observed lower BBR severity (P = >0.05). Similar results were also obtained for the incidence of BBR, for which there appeared to be a weak correlation with the amount of pruning weight obtained at the end of the season (P= 0.078) for the flowering treatment, with similar results obtained for the PBC treatment.

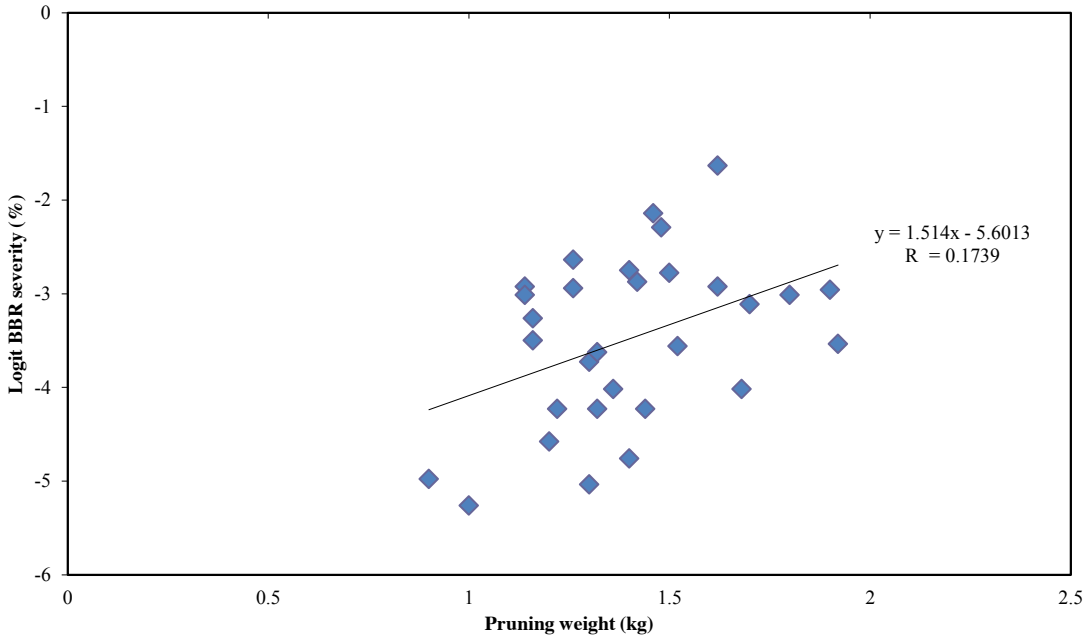

Figure 5.20: Scatter plot showing a weak correlation between pruning weight and logit BBR severity in the flowering treatment (n = 60).

In order to determine the effect of vine vigour on BBR severity spatially, the block was divided into five sections according to the PCD imagery (Figure 5.16). These sections were

low vigour (red), low-medium (yellow), medium (green), medium-high (aqua) and high vigour (blue) for both treatments. Fungicide treatment and vine vigour were found to be independent and significant factors associated with BBR severity on the 3rd April (Table 5.10). However, there was not a steady increase in BBR severity as vigour increased for the April 3rd data (Table 5.10). The vigour section categorised as 'high' had the highest BBR severity across treatments, although it was similar to the BBR severity for the 'medium-high' category for the flowering treatment. ANOVA analysis within the flowering treatment found that there was a trend in BBR severity in which both higher and lower vigour vines had higher severity levels, whereas the medium vigour vines had lower severity ($P = 0.069$). The effect of vine vigour on sporulation was also found to be independently significant with no significant interaction with treatment with the higher vigour vines having the greater amount of sporulating *B. cinerea* (Table 5.11). Vine vigour was found to be independently a significant factor in BBR incidence for both the March 24th assessment ($P = 0.001$) and slightly less so for the April 3rd assessment ($P = 0.057$) (refer to Table 5.12 and 5.13). Data showed that both the medium to high and high vigour categories overall were associated with higher incidence of BBR for both treatments. There was no significant interaction between vine vigour and fungicide treatment.

Table 5.10: Mean BBR severity (%) according to vine vigour and fungicide treatment as of the 3rd April 2009. An unbalanced ANOVA was completed using logit-transformed figures (in parenthesis) (residual df = 290). The P values and least significant differences (lsd- in parenthesis) are as followed: - vigour P = 0.016 (0.3951); fungicide treatment P = <0.001 (0.2462); interaction P = 0.349. The lsd is represented in the table via letters next to the mean values, where letters are the same it shows that there is no significant difference between the values.

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	3.01 (- 3.86)	0.94 (- 5.04)	1.97 (- 4.45) b
Low - Medium	3.95 (- 3.61)	1.86 (- 4.46)	2.91 (- 4.04) c
Medium	2.19 (- 4.30)	1.08 (- 4.72)	1.63 (- 4.51) b
Medium - High	4.05 (- 3.55)	1.45 (- 4.59)	2.75 (- 4.07) c
High	3.94 (- 3.57)	2.37 (- 4.31)	3.16 (- 3.94) a
Mean	3.44 (-3.77) a	1.49 (- 4.64) b	

Table 5.11: Mean percentage of sporulating *B. cinerea* (%) according to vigour and spray treatment on the 3rd April 2009. An unbalanced ANOVA was completed using logit-transformed figures (in parenthesis) (residual df = 290). The P values and least significant differences (lsd- in parenthesis) are as followed: - vigour P = 0.008 (0.3996); fungicide treatment P = <0.001 (0.2490); interaction P = 0.720. The lsd is represented in the table via letters next to the mean values, where letters are the same it shows that there is no significant difference between the values.

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	2.12 (- 4.30)	0.59 (- 5.40)	1.34 (- 4.85)
Low - Medium	2.96 (- 3.95)	1.27 (- 4.89)	2.11 (- 4.42)
Medium	1.73 (- 4.52)	0.65 (- 5.22)	1.19 (- 4.87)
Medium - High	3.32 (- 3.85)	0.88 (- 5.08)	2.10 (- 4.46)
High	3.36 (- 3.79)	1.67 (- 4.71)	2.52 (- 4.25)
Mean	2.71 (- 4.08) a	0.97 (- 5.08) b	

Table 5.12: Mean percentage incidence of BBR (%) according to vine vigour category and fungicide treatment using assessment taken on the 25th March 2009. Results from an unbalanced ANOVA is also shown (residual df = 290). The calculated *P* values and least significant difference (lsd) are as follows: - vigour *P* = 0.001 (7.846); fungicide treatment *P* = <0.001 (4.889); interaction *P* = 0.347). The lsd is represented via letters against the mean where P = <0.05. Where the letters are the same, there is no significant difference between the factors. The lsd ranking is shown for both fungicide treatment columns and the vigour mean column.

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	37.32	24.44	30.88 b
Low - Medium	44.94	30.95	37.94 a b
Medium	38.04	32.56	35.30 b
Medium - High	55.76	34.52	45.14 a
High	47.86	34.68	41.27 a
Mean	45.24 a	31.37 b	

Table 5.13: Mean percentage incidence of BBR according to vigour and fungicide treatment for the 3rd April 2009. Results from an unbalanced ANOVA is also shown (residual df = 290). The calculated *P* values and least significant difference (lsd) are as follows:- vigour *P* = 0.057; fungicide treatment *P* = <0.001 (4.905); interaction *P* = 0.306. The lsd is represented via letters against the mean where P = <0.05. Where the letters are the same, there is no significant difference between the factors.

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	43.69	20.28	31.99
Low - Medium	43.27	28.97	36.12
Medium	33.83	27.46	30.64
Medium - High	46.67	30.00	38.34
High	49.33	34.38	41.86
Mean	43.43 a	27.96 b	

The repeated measures analysis found that vine vigour was independently a significant factor in BBR severity with the medium- high to high vigour vines having greatest severity. There was a significant correlation with time and the vigour category, but the analysis did not find a

significant correlation between time, vigour category and fungicide treatment (Table 5.14, Figure 5.21).

Table 5.14: Summary of repeated measures analysis for the effect of treatment and vigour classification according to PCD category and BBR severity at different assessment dates.

Source	df	SS	MS	F value	P > F
Vigour Category	6	34.3661	5.7277	2.50	0.023
Fungicide Treatment	1	8.8939	8.8939	3.88	0.05
Vigour Category.Fungicide Treatment	2	6.3383	3.1691	1.38	0.252
Residual	290	664.5495	2.2916	4.18	-
Time	3	983.1768	327.7256	597.82	<0.001
Time.Vigour Category	18	78.7166	4.3731	7.98	<0.001
Time.Fungicide	3	44.029	14.6676	26.76	<0.001
Time.Vigour Category. Fungicide	6	4.1215	0.6869	1.25	0.203
Residual	870	476.9337	0.5482	-	-
Total	1199	2301.0993	-	-	-

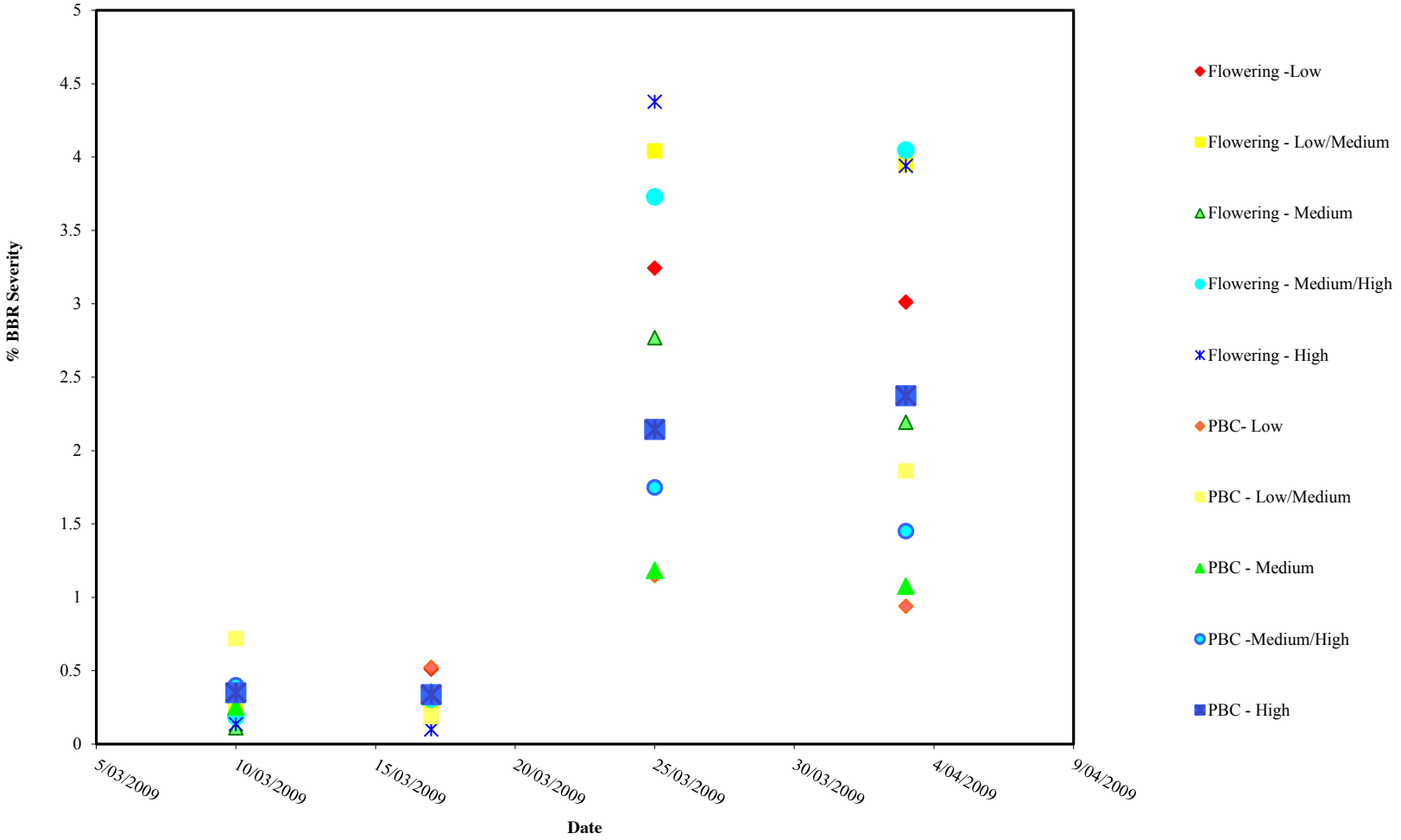

Figure 5.21: Temporal progression of BBR severity (%) over time according to vigour category for vines subjected to flowering and PBC spray timing. Vigour category includes Low, Low- Medium, Medium, Medium- High, and High as determined by PCD image in Figure 5.17. Colour palate is based on that as used in the PCD imagery where low vigour (red tones), low-medium (yellow tones), medium (green tones), medium-high (aqua tones) and high vigour (dark blue tones).

5.3.8. Fruit ripening & juice characteristics

Fruit ripening varied slightly across the block (data not shown), and there was no significant difference between clones (Appendix F, Figure F1). The commercial harvest was in two stages with half the block harvested in March for sparkling wine (in the rows where there were no tagged vines) (19/03/2009) and the rest for table wine (harvest date: 8th April 2009). The block with tagged vines (table wine block) was harvested when the fruit reached an average of 23.4° Brix (13 Bé).

Titratable acid (TA) and pH were not significantly different among clones, although Brix (total soluble solids) was marginally significant (P = 0.051, residual df = 53). There was no significant interaction between treatment and clone for any of the juice parameters (Table F4 in Appendix F). There was a significant difference in TA attributable to treatment (Table F6 in Appendix F). There was a significant interaction between treatment and vine vigour category (refer to section 5.3.7 for vigour explanation) for juice pH (P = > 0.001) (Table F7, Appendix F).

5.3.9. The application of qPCR to quantify B. cinerea in berries

5.3.9.1. Total DNA yield from DNA extraction

DNA yield varied across the 300 DNA samples (150 per treatment). For the flowering treatment, 48 samples were below the detectable level of 39.073 pg/µL and therefore assigned the arbitrary concentration of 1 ng/µL. Of the rest, the mean DNA concentration was 2.9 ng/µL, ranging from 1.78 ng/µL (vine 140) to 15.3 ng/µL (vine 84). For the PBC treatment, all samples had quantifiable amounts of DNA, with a mean of 3.4 ng/µL. The minimum amount of DNA recorded was 0.2 ng/µL (vine 142); two samples had relatively high concentrations; 19.48 ng/µL (vine 129) and 11.56 ng/µL (vine 138), and the remainder were below 5 ng/µL.

5.3.9.2. Standard dilution series

Out of the 16 batches of samples assayed by qPCR, regression analysis for the standard curve showed that run 2 was significantly different to runs 10 (P = 0.049) and 16 (P = 0.02), and run 14 was significantly different to run 16 (P= 0.046). There was no significant difference between the other qPCR runs (P = 0.05). The R^2 values for each set of standards ranged from 97.5 to 99.6 (Table F8, Figure F2 Appendix F). The reaction efficiencies calculated for each run using the dilutions series ranged from 82% to 112%, with most runs between 95 - 99% (Table F7, Appendix F). The optimal range is between 90-100%, reflecting, that after each cycle the amount of PCR produced doubles.

5.3.9.3. Field samples

Three samples failed the qPCR assay with no Ct value recorded for either *B. cinerea* or *V. vinifera* DNA. The samples that failed included one sample from the PBC treatment (vine 38) and two from the flowering treatment (vines 3 and 88). No *B. cinerea* DNA was detected in 29 samples from the flowering treatment and 27 samples from the PBC, but these results were deemed valid as *V. vinifera* DNA was detected in the samples. A trend was evident for the PBC treatment to have less *B. cinerea* DNA content and percentage *B. cinerea* DNA than the flowering treatment (Table 5.15), but there was too much variability for this to be a significant difference between the treatments.

Out of the 297 samples in total, 36 samples from the flowering treatment and 20 samples from the PBC had *B. cinerea* DNA amounts within the range of the standards (minimum of 350 fg/reaction (Table 5.16 for summary data). Chi-squared analysis of the samples with ≥ 350 fg/μL (with the failed samples removed) showed that the flowering treatment had significantly more samples with detectable *B. cinerea* (Table 5.17, P = 0.028 for a likelihood chi-square value from a 2 × 2 contingency table). Chi-squared analysis of all samples in which the *V. vinifera* control was amplified (278 samples) showed that although the flowering treatment resulted in more samples with ≥ 350 fg, it was no longer significant at P = 0.05 (table not shown). Using the Mann Whitney U test, there was also no significant difference between the treatments

(P = 0.304). However, the Mann Whitney U test using the two sample pools suggested that there was a trend, which showed that the flowering treatment resulted in greater amounts of *B. cinerea* DNA and higher percentage DNA in the grape samples than the PBC samples.

Investigation into the effect of clone on the amount of *B. cinerea* DNA detected in the samples found that there was a significant interaction between fungicide treatment and clone for percentage *B. cinerea* DNA. The Penfolds clone resulted in a higher *B. cinerea* percentage DNA than the other two clones (G9V7 and I10V1) for the flowering treatment only (P = 0.04) (Table F9, Appendix F).

Table 5.15: Summary of qPCR results for the quantification of *B. cinerea* DNA (pg/ reaction) in 50-berry samples, excluding failed results, and the percentage *B. cinerea* DNA within each of the 297 samples.

	Flowering		PBC	
	B. cinerea DNA Amount (pg/reaction)	% *B. cinerea* DNA	*B. cinerea* DNA amount (pg/reaction)	% *B. cinerea* DNA
Number of Values	148		149	
Mean	1.937	0.0284	1.074	0.0156
Median	0.079	0.0016	0.050	0.0007
Minimum	0	0	0	0
Maximum	221.000	2.4690	112.300	0.8980
Lower quartile	0.021	0.0003	0.018	0.0002
Upper quartile	0.286	0.0005	0.111	0.0038
Standard deviation	18.180	0.2070	9.228	0.0851
Standard Error	1.494	0.0170	0.756	0.0070
Variance	330.300	0.0427	85.216	0.0072
Sum of Values	286.700	4.1980	160	2.3240

Table 5.16: Summary of the amount of *B. cinerea* DNA amplified and percentage *B. cinerea* for the samples above the limit of detection of 350 fg/ reaction (0.350 pg).

	Flowering *B. cinerea* DNA Amount (pg)	% *B. cinerea* DNA	PBC *B. cinerea* DNA Amount (pg)	% *B. cinerea* DNA
Number of Values	36		20	
Mean	7.750	0.112	7.613	0.103
Median	0.787	0.021	1.339	0.028
Minimum	352.700	0.004	0.383	0.006
Maximum	221.000	2.469	112.300	0.898
Lower quartile	0.600	0.010	0.805	0.014
Upper quartile	1.437	0.039	3.091	0.051
Standard deviation	36.630	0.412	24.730	0.217
Standard Error	6.104	0.069	5.529	0.049
Variance	1341.000	0.170	611.300	0.047
Sum of Values	279.000	4.035	152.300	2.052

Table 5.17: Contingency table showing the number of samples with at least 350 fg DNA and the proportion of samples that had less than the 350 fg (0.350 pg). Samples exclude failed results.

	Number of samples with the indicated amount of *B. cinerea* DNA (fg)		
Treatment	< 350 fg (%)	≥ 350 fg	Number of Samples
Flowering	112 (76 %)	36 (24 %)	148
PBC	129 (87 %)	20 (13 %)	149
Pearson Chi-square value	4.74		
P Value	0.030		
Likelihood chi-square value	4.82		
P Value	0.028		

5.3.10 Environmental data for field trial

5.3.10.1. Temperature

Comparison of data output from the weather station and ibuttons, by co-locating the ibuttons with the data logger sensors, was not completed; hence, only ibutton data were used for within-block comparisons. The mean growing temperature (January-April) was slightly warmer in the top half of the block, which appeared to correlate with increased vine vigour (Figures 5.22; Table F10, Appendix F). Overall, the western side of the block was warmer than that of the southern end (towards the main weather station). The *t* test results conducted between the eastern (lower vigour) and western side (higher vigour) of the block showed that only in March was there a slight

significant difference between the two sections, with the western side being warmer (P= 0.055).

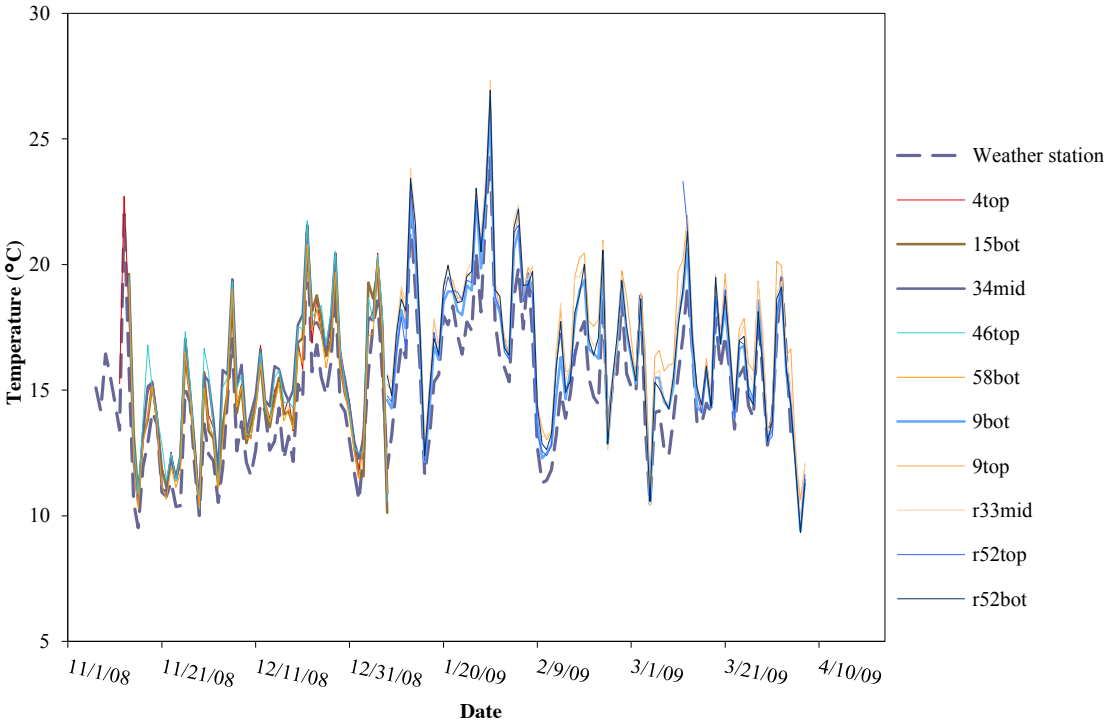

Figure 5.22: Mean daily temperature collected from the main weather station and each of the ibuttons placed in the canopy. Weather station data were collected from 7th November 2008 to 4th April 2009; ibutton data were collected from 12th November 2008 to 7th April 2009. Note that the locations of the ibuttons changed on the 8th January 2009 (R4, R15, R34, and R46)

5.3.10.2. Relative humidity

Overall relative humidity was generally less than 90% (Figure 5.24, Table F10, Appendix F). On the few occasions when relative humidity was greater than 90% [three periods during flowering stage (November- December) and four periods during berry ripening phases (February- March)], it lasted for a short period at night or early morning during cooler weather (Figure 5.23, Table F11 Appendix F). Statistical analysis of the RH data found no significant differences in data output among the ibuttons.

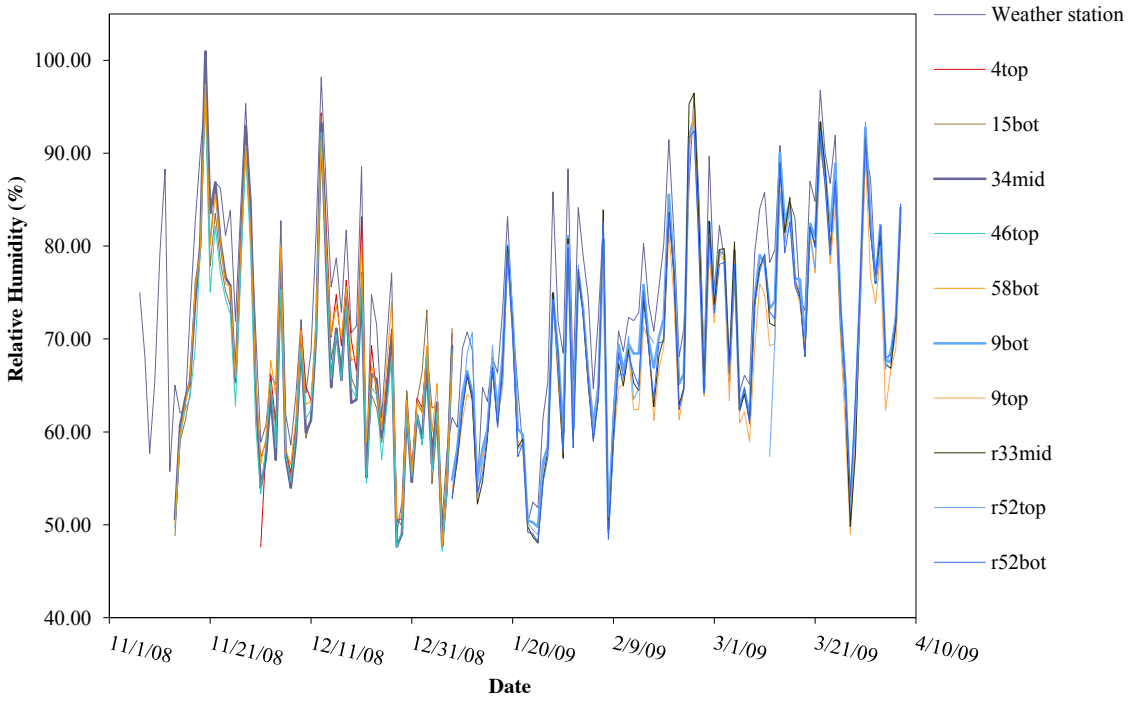

Figure 5.23: Mean daily relative humidity (%) for the weather station near the trial block and ibuttons placed in the canopy of the trial block.

5.3.10.3. Rainfall

There were 74 rainfall events recorded during the growing season from 7th March 2008 to 4th April 2009. Of these, four rainfall events resulted in more than 17 mm. The first two were during the flowering in November 2008 and the other two were during the later stages of ripening in February and March close to harvest (Figure 5.24). However, there were long periods between the rainfall events, enabling bunches and canopy to dry out.

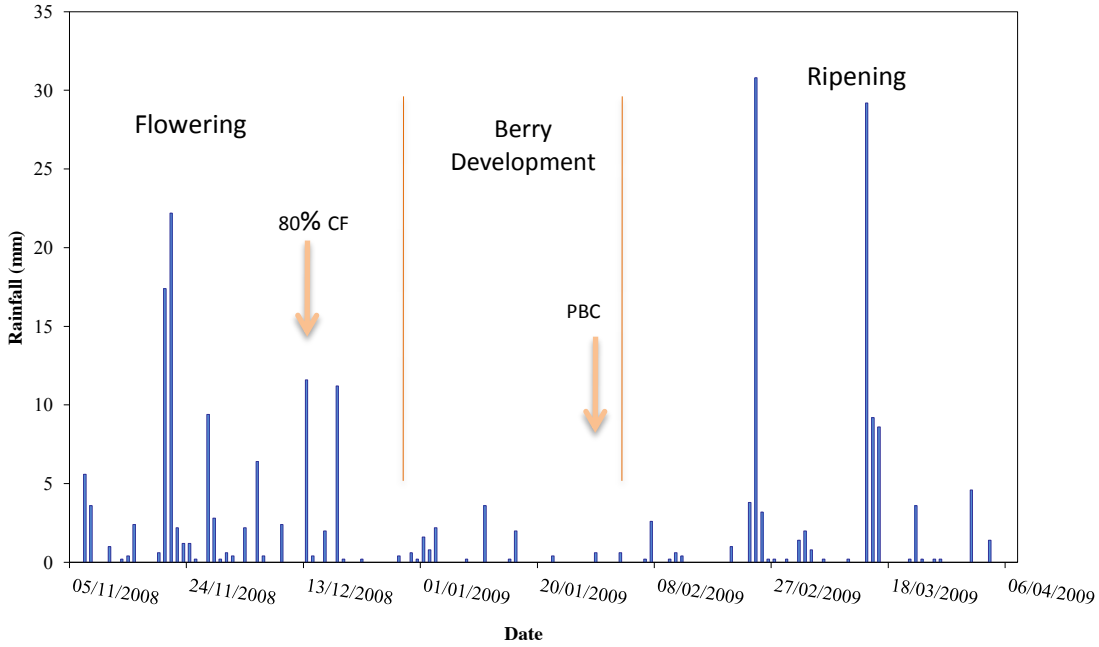

Figure 5.24: Total daily rainfall recorded by the main weather station at the trial site for the 2008- 2009 trial season. Fungicides were applied either on 15th December (80% capfall) or 27th January (pre-bunch closure). Also shown is the period where flowering, 80% capfall (CF), berry development, pre- bunch closure (PBC) and ripening occurred during the season.

5.4. Discussion

Conducting an experiment over an entire vineyard block allowed spatial variation in disease severity to be taken into account when comparing two different spray programs for management of BBR. This approach also allowed for the use of commercial vineyard spray equipment, which is unachievable in small plot trials (Crisp *et al*. 2006). The results provided further evidence of the utility of whole-of-block experimentation. Previous spatial experiments have investigated effects of management practices on grape yield and quality (Bramley and Lanyon 2003; Bramley and Hamilton 2004; Bramley 2005; Bramley *et al*. 2005b; Bishop and Lark 2006; Bramley 2007; Panten and Bramley 2007; Panten *et al*. 2010; Panten and Bramley 2011). Unlike previous studies, spatial variation in vine vigour, soil moisture, slope or clone, was compared with spatial variation in BBR severity at different times during the pre-harvest period to identify any associations.

Spray timing is an important component in the effectiveness of crop protection. The present study found that the mean BBR severity under the flowering treatment was approximately double that of the PBC treatment. Even though these results support the findings of Edwards *et al*. (2009), the study highlighted the risk of conducting small-plot trials as they may not fully reflect the commercial environment, as different areas of the block may respond differently. The study by Edwards *et al*. (2009), using small-plot trials in cool climate viticulture regions, found that mid-season sprays were more effective than early season sprays, and were equivalent in effectiveness to a full season spray program, under conditions of low to moderate disease risk. The whole-of-block experiment showed that BBR severity varied in space and time for both treatments. Comparison of severity on 24th March with severity on the 3rd April showed a greater proportion of the block had no significant difference between treatments at the earlier date, especially towards the bottom of the slope (eastern side). By harvest, however, the PBC treatment was found to be more effective over a wider area in reducing BBR severity. Zitter & Wilcox (2007b) conducted a glasshouse experiment to investigate the efficacy of commercial fungicides applied at fruit set, PBC and *véraison* after which bunches were inoculated with *B*. *cinerea* strain to determine the extent of internal colonisation by this fungus. Their results indicated that mid-season sprays prevented the fungus from internal colonisation after this period. The results of the present study are consistent with the findings of Zitter & Wilcox (2007b) and suggest that an effective residue or fungicide dose persisted long enough to prevent substantial colonisation of the ripening berries.

The ONFIT results, which can indicate how much latent infection was present at PBC, although not significant because the values were very low, showed that the flowering treatment had half the incidence of infected berries than the PBC treatment. Taking into consideration the end of season BBR severity and incidence results, it could be postulated that fungicide applied at PBC may have resulted in the mortality of some of the latent infections that had established between flowering and early berry development, based on the findings of Zitter & Wilcox (2007b). However, this result is not conclusive as the decrease in *B*. *cinerea* incidence could have also been due to natural mortality in conjunction with fungicide. Further studies, investigating incidence of latent *B*. *cinerea* colonisation at flowering and *véraison* would provide greater detailed into establishment of the fungus, which was not explored during this

field trial. Previous studies using ONFIT have shown that it can be a useful tool in predicting potential BBR risk under certain situations where there is high infection numbers (Beresford and Hill 2008). In this study the ONFIT results showed low infection numbers overall, with expression of latent infection lower than that of the PBC treatment. These results did not reflect the final BBR severity at harvest, where the mean severity and incidence of BBR for the PBC treatment was lower. This finding supports the hypothesis that the increase in severity in the flowering treatment was resulted from post flowering infection and potentially from single infection events via wounds in the berries.

The spatial analysis of the flowering treatment temporal data suggested that the increase in BBR severity at each assessment date was attributed to berry-to-berry spread and not that of new infections or secondary spread. It can be observed for this trial that the severity of BBR at this site is due to fungal growth after latent infection or via a single direct infection event (via wounds such as splits in berries) in one or more berries, followed by berry-to-berry spread within the bunch. Due to the high rate of berry splitting that occurred at this trial site, direct infection of berries was a possible infection pathway. In order to map latent infection accurately, multiple samples in time and space would have been required, which was beyond the scope of the current study. Further investigations over a number of seasons are warranted to determine conclusively if latent infection or direct infection, followed by berry-to-berry spread, is the main pathway for *B. cinerea* infection and development of BBR at this vineyard and elsewhere. Otherwise, this pathway may be the result of seasonal conditions and other pathways may dominate in other seasons.

Previous studies have shown that the temporal progression of BBR in small plots can be successfully modelled (Beresford *et al*. 2006). Using the variogram generated for the flowering treatment on the 3rd of April, five different epidemics were characterised for the area under study, whereas three were characterised from the variogram for the PBC treatment. The different epidemics were identified by setting the severity thresholds in the legend parameters within the GIS software, which is controlled by the operator. Too many categories may create a more detailed but confusing map, while not enough categories may not generate any beneficial information (no significant differences between treatments or locations within block).

The distinction of different epidemics for a single treatment was supported by statistical analysis of the AUDPC.

The flowering treatment showed that the progression of BBR severity could vary within a block; in this case, two of the epidemics reached a final BBR severity above that of the overall mean. Spatial mapping according to severity thresholds might also provide information for future seasons, highlighting the higher disease risk zones, enabling a more tailored approach to control and monitoring in seasons conducive to BBR, as well as harvesting decisions. The spatial analysis at this point also showed what other factors might contribute to BBR severity, which was further examined in this study. Similar results as discussed above were reflected in the PBC results, but not to the same extent as the flowering treatment.

Managing vine vigour is an important component of disease management in the vineyard (Gubler *et al*. 1987; Fermaud *et al*. 2007, Valdés-Gómez *et al*. 2008). This trial suggested that there appeared to be a correlation between BBR severity and vine vigour, measured as PCD the following season. The PCD index has been used in previous studies as a measure of vine vigour as there is little season-to-season variation in PCD measurements (Bramley and Hamilton 2004; Acevedo-Opazo *et al*. 2008; Bramley *et al*. 2011). As PCD is a measure of active canopy growth, it can be used to determine high disease risk zones. Canopy density affects the microclimate surrounding the grape bunches, which will influence the development of disease according to the duration of surface moisture, fungicide penetration and dose, and air penetration (wind) (Gubler *et al*. 1987; Fermaud *et al*. 2007). Variation in vine vigour across the block can be attributed to a number of factors including the three different clones within the block, as well as soil variation (not explored in depth during this trial). Clone G9V7 was present in the lower vigour area and this correlated with lower disease severity, compared with higher disease severity for the two other clones where vigour was higher. Clone G9V7 is known to be a less vigorous Chardonnay clone than Clone I10V1 (Cirami and Ewart 1995) and this was supported by the PCD and trunk diameter data. Pruning weight is a measure of grapevine growth for that season, which can be used as a tool to measure overall vine vigour. The results in the flowering treatment showed that there was a weak positive correlation between pruning weight and BBR severity and incidence, supporting what was observed with

the PCD analysis. However, it should be noted that the analysis only used 30 vines due to a time constraint. These preliminary results showed that further investigation at a larger scale is needed to determine the full extent of the correlation between cane weight as a vigour measure and BBR severity, due to the nature of grapevine growth variation. It can be also noted that cane length and width will vary within varieties and clones, as well regional and seasonal factors, warranting a bigger survey in order to truly understand the potential relationship.

The temporal curves generated for each of the treatments showed that both vigour and time were significant factors in disease development, since vines of both high vigour (dense canopies) and low vigour were found todevelop more severe BBR than those of moderate vigour, according to the repeated measures analysis. By using imagery such as PCD to define vigour zones, there is the potential for growers and contracting wineries to use the information to make strategic decisions about management, fruit grading and harvesting (Bramley and Hamilton 2004; Bramley *et al*. 2005; Bramley 2005; Proffit *et al*. 2006). The use of the vigour maps could identify potential disease hotspots that need the most attention. For contracting wineries, the map may provide information leading to selective harvesting or 'block splitting' to provide different fruit grades as required, instead of the whole block being graded into one category. The information gained from spatially mapping PCD at a chosen point in time during the ripening phase (*véraison*) has been demonstrated here to have potential for influencing management strategies for future seasons, to reduce BBR risk and therefore improve fruit quality. Although BBR severity and vine vigour were higher on the upslope of the block (western side), there was no correlation with the EM38 readings. The elevation survey suggested a trend with PCD and BBR severity, which was not conclusive due to the study being completed using dGPS. For accurate elevation studies and spatial mapping RTKGPS is preferred due to the lower error that is involved (Proffit *et al*. 2006). If RTKGPS was used analysis of the relationship between the elevation of the block, BBR severity and vigour could have been explored further.

Weather plays an important role in driving the epidemics of BBR, especially the severity of disease symptoms. As stated previously, overall mean BBR severities for both treatments were < 5%. The weather for this season was not highly favourable

for the development of severe BBR, as it was a relatively dry season with only minor wet weather events towards the end of the season. BBR is a disease favoured by high relative humidity (English *et al*. 1989; Broome *et al*. 1995; Beresford 2007). The relative humidity readings recorded in the canopy and the site weather station were generally below optimal RH for BBR development (95% or higher for a period of at least 15 hours according to Gubler *et al*. (1987); Nair *et al*. (1988); Thomas *et al*. (1988); Nicholas *et al*. (1994); Vail *et al*. (1998)). Studies by Zitter & Wilcox (2007a) have shown that the proportion of established latent infections that continue to grow during the season is proportional to the relative humidity in the bunch zone. In this study, the environmental data collected showed considerable variation in relative humidity across the block with no apparent correlation with vine vigour or disease severity. However, canopy temperature was higher in the higher vigour zones, from the middle of the block and up the slope, which correlated with increased disease severity. In addition, soil moisture was higher in these zones and it was observed that the ground was often muddy in these locations compared to the other zones. The soil in this region was a clay loam, with better water holding capacity and texture than the silty soil further down the slope where BBR severity was less. The dry weather resulted in very low disease expression at the first two assessment dates. It was not until later in the season when BBR severity increased at the last two assessment dates, coinciding with moist weather conditions from significant rain events. The rain events which also occurred during the flowering- early berry development may have resulted in the high amount of splitting that occurred, which probably caused the high percentage of other rots found, which may have impacted the scoring of BBR. The splits in some sections did eventually dry out; where they did not, other rots were distinguishable and the characteriscally pink-brown rot of BBR were observed. These other rots, may have impacted the colonisation of BBR, due to competition for space and nutrition.

The weather for this particular season at this particular site demonstrates the role of favourable weather in BBR development and that effectiveness of fungicide timing in controlling BBR is only part of the equation. In recent years, there has been a push by researchers to develop predictive models for BBR risk, in the hope of enabling growers to develop a more strategic approach in disease management (Broome *et al*. 1995; Balasubramaniam *et al*. 2000; Beresford *et al*. 2006; Beresford 2007). The

main tool used in these models is the collation of weather data from previous and current seasons to determine the in-season risk of infection (Broome *et al*. 1995; Beresford *et al*. 2006; Beresford *et al*. 2007). With this in mind for the season under study, hypothetically the risk of infection may have been very low during the key period of latent infection establishment. However during this trial, it was not possible to accurately identify the key infection stages, as the trial relied on natural *B*. *cinerea* population to become established.

The qPCR technique applied in this study was found to be successful in determining the amount of *B*. *cinerea* DNA in naturally infected grape berries. The study found that a greater proportion of samples from the flowering treatment had higher levels of *B*. *cinerea* DNA above the 350 fg limit than that of the PBC treatment. However due to the large variation in *B*. *cinerea* amount quantified in the DNA samples across the two treatments, the statistical analysis was unable to distinguish between the treatments, although analysis did show that a greater proportion of the samples in the flowering treatment had higher amounts of *B*. *cinerea* DNA than the PBC samples. There was a significant interaction between treatment and clone, supported by the visual assessments, with the Penfolds clone resulting in higher BBR levels. To my knowledge, this is the first trial testing the qPCR assay on a large scale. Previous field-based studies used small plot trials with smaller sample numbers or samples collected for the purpose of assay development (Cadle-Davidson 2008; Celik *et al*. 2009; Diguta *et al*. 2010).

5.5. Conclusion

This study has highlighted the use of whole blocks as an important experimental tool for use in disease epidemiological studies. It has the potential to rapidly provide information to both researchers and industry that is more useful and realistic than from small-plot trials. The PBC spray was found to be more effective in minimising BBR than the flowering treatment, supporting the previously published small plot trials of Edwards *et al*. 2009 and Evans *et al*. 2010b. This study demonstrated that within a vineyard block there is variation in BBR severity spatially across the block. It also showed that vine vigour could have an impact on severity with vineyard sections showing high PCD resulting in a greater BBR severity over time. The

investigation into the relationship of clone and BBR found that it could have a significant impact on BBR severity, although the results also suggested that it may be the interaction between clone and vineyard site that is important. The study also found that the spatial variation occurring in vine vigour was found to have a significant effect on BBR severity. Pruning weight as a measure of vine vigour was also found to correlate with BBR severity to some extent, warranting further investigation on a larger scale. The application of the qPCR technique was successful in determining the load of *B. cinerea* in the fruit at harvest, with a trend of higher amounts of *B. cinerea* DNA found in the berries of the flowering treatment than the PBC treatment. The qPCR results also complemented the visual assessment, with the latter indicating that Chardonnay clone was an important factor associated with BBR severity, along with fungicide treatment.

Chapter Six
General Discussion

Botrytis bunch rot, caused by *B. cinerea*, is one of the most economically important diseases in the wine industry with an estimated cost of $52 million per annum for the Australian industry (Scholefield and Morison 2010). This cost estimate does not include the extra labour, equipment and materials that might be required to implement pre-harvest disease assessment, selective harvesting, fruit sorting or remedial winemaking (N. Fryar, Jansz winemaker, personal communication). The focus of disease management must be to implement effective preventative measures so that disease development and these extra costs are minimised. This project investigated the epidemiology of this disease focusing on detection methods and vineyard practices. Five broad themes were investigated in this study. These were:

1) Development and application of qPCR to detect and quantify *B. cinerea* DNA in both grape and juice samples,

2) Comparison of mid-infrared spectroscopy, ELISA and qPCR in estimating levels of *B. cinerea* in juice from different treatments investigating spray timing in a small-plot field trial,

3) Characterisation of the temporal progression of BBR epidemics in small plots and spatio-temporal progression of BBR epidemics in whole vineyard blocks,

4) Investigation of possible infection pathways of *B. cinerea* in the Tasmanian field sites studied, and

5) Investigation of vine and environmental factors within a single vineyard block contributing to BBR incidence and severity.

In investigating these broad areas, this PhD research was able to demonstrate the first application of 'whole-of-block' experimentation to study the spatio-temporal development of BBR, with the work conducted under the guidance and in collaboration with Dr Rob Bramley, CSIRO.

The first theme, covering the development and application of qPCR to detect and quantify *B. cinerea* in grape samples, currently fits with industry needs for novel, objective and rapid methods for assessing fruit quality. In this case, cost-effective and accurate assessment of fungal contamination is needed to overcome the deficiencies associated with visual disease assessment. The application of qPCR in this study highlights both the benefits and limitations of the use of a molecular tool for research purposes. During the adoption of this technique, this study was able to build on the work previously published by Cadle-Davidson (2008) in which was used to detect *B. cinerea* colonisiation in asymptomatic berries. However, the process of adoption ultimately led to the design of a new duplex assay that could detect *B. cinerea* and *V. vinifera* DNA simultaneously. The assay was found to be useful in determining the amount of *B. cinerea* DNA in both grape berry and juices samples; however, the variable results generally prevented detection of statistically significant differences among treatments or sites. Further optimisation of the assay and an improved sampling strategy might result in greater reliability and repeatability of results.

Temporal disease progress curves derived for the study of BBR epidemiology have relied on observing incidence or severity via visual scoring (Beresford *et al.* 2006). To observe latent infection, the host tissue needs to be processed according to ONFIT when the tissue shows no sign of infection (Holz *et al*. 2003; Cadle-Davidson 2008). One component of this project was to build on the work of Cadle-Davidson (2008) to use qPCR as a tool to track the colonisation of *B. cinerea* from early berry development (PBC), as latent infections are potentially established at any time before harvest (Emmett *et al*. 2005). The results of this study suggested that the fungus infected during the early stages of berry development at the field site studied, but that the level of colonisation, as indicated by the amount of *B*. *cinerea* DNA, remained at a similar level through to harvest. There was no correlation with changes in BBR severity, as any increase in *B*. *cinerea* DNA was not statistically significant. The sampling strategy and the fact that only one season of data was collected indicates that further investigation is warranted. Moreover, *B. cinerea* is a necrotroph, which means that it colonises dead tissue by releasing enzymes in advance of its colonisation. Indeed, the ripening phase presents senescing tissue suitable for colonisation by this fungus (Pashkoulov et al. 2002; Elmer and Michailides 2004;

Williamson *et al*. 2007). A detailed experiment is needed to understand the link between fungal colonisation (DNA amount) and the symptoms expressed in relation to the biochemical changes that occur due to the fungus releasing enzymes and the natural senescence of the grape berry. Changes in host tissue quality (cell number and volume; chemical composition; living, senescent or dead tissue) relative to the extent of fungal colonisation need to be quantified concurrently with application of qPCR to understand how these changes might impact on the amount of non-degraded target DNA that can be amplified, and, ultimately, the true extent of colonisation by *B*. *cinerea*. How this research could be designed remains a problem to be solved.

Application of qPCR using samples from a whole vineyard block indicated that this technique was able to detect differences between spray treatments, with the flowering spray timing having both higher amounts of *B. cinerea* DNA and greater BBR severity than the PBC spray timing. In this regard, qPCR may be very useful for detecting differences between treatments at a single point in time. There is also the potential to use qPCR to determine incidence level of samples containing *B*. *cinerea* DNA above a pre-determined threshold amount and in certain situations could be more appropriate than relying on the absolute amount of DNA quantify. This would then take into the consideration the influence that tissue decay from both BBR and other rots as well as changes in berry composition would have on the amount of *B*. *cinerea* DNA quantified by the assay. This type of analysis was conducted (Chapter 5) and it resulted in statistical separation of treatments. For an industry application, incidence of disease is more widely used by companies, when fruit quality is suspect due to bunch rots, rather than solely relying on a mean severity. The qPCR test could then be used in this way to gauge a potential BBR incidence risk of the disease earlier in the season, with a follow up assessment if the disease progresses to above contracting thresholds. Time constraints meant that samples collected in the whole of block experiment at earlier crop stages were not assayed; therefore, it was not possible to study the spatio-temporal changes in *B*. *cinerea* DNA across the vineyard block.

Given the industry need for rapid and accurate quantification of fungal diseases, an experiment was conducted using grape juice samples to compare qPCR, ELISA and MIR (mid-infrared) spectroscopy to measure BBR load in comparison with visual

severity scoring. To date the investigations into the use of spectroscopy for determining BBR levels has been limited (Cozzolino *et al*. 2003, Versari *et al*. 2008, Scott *et al*. 2010). The MIR wavelength has been applied successfully in a study by Versari *et al*. (2008) that measured gluconic acid and glycerol, which are by-products of *B*. *cinerea* infection. The MIR results in this PhD study were found to correlate weakly with the observed BBR visual severity for each treatment, as were the amounts of *B. cinerea* DNA. However, there was a trend which showed that the samples from the mid-season fungicides, in comparison with nil and early fungicides, had less DNA and lower BBR severity at harvest relative to other treatments. The fact that there was a weak correlation for the MIR results with visual severity show that there is the potential for it to become a readily accessible alternative tool for industry and research, once standard methods have been developed. There is also the potential to explore other wavelengths such as NIR, which are readily used in wine and grape analysis (Cozzolino *et al*. 2007b; Gishen et al. 2010). The ELISA test was the only assay that allowed spray timing treatments to be separated statistically based on the SI (signal intensity) scores for juice samples. The limitations of ELISA were demonstrated in a preliminary experiment where it failed to work when applied to berry samples collected at pre-bunch closure. Nevertheless, application of ELISA might be useful for harvest assessments of treatments in which bunch rots are caused by multiple microorganisms and when it is difficult to ascertain which parts in a rotten bunch are caused by *B*. *cinerea*.

At the time of this study, there were no known published records in which qPCR was used to determine the amount of *B. cinerea* DNA in grape juice samples, but there are examples in which qPCR has been used for assessing wine contaminants (Gindreau *et al.* 2001; Delaherche *et al*. 2004; Culbert *et al*. 2008) and powdery mildew (Stummer *et al*. 2006). Quantitative PCR was shown to be succesful in determining the amount of *B. cinerea* DNA; however, the results were not directly comparable to the ELISA or MIR tests or visual assessment scores given that each test is measuring a different characteristic of BBR, i.e qPCR- DNA, ELISA- botrytis antigens, MIR- wavelengths. This was a preliminary investigation into the use of grape juice samples as a source for DNA extraction rather than the time consuming method of grinding frozen berries with a mortar and pestle. The use of juice rather than whole berries could have been applied to all grape samples from PBC onwards, as they could, in theory, have been

homogenised rapidly using specialised equipment based on a method designed for tannin analysis in grape samples (AWRI 2009).

Both years in which field trials were conducted (2007-08 and 2008-09) resulted in relatively low BBR incidence and severity, reflecting the mostly dry conditions that were observed, especially in the 2007-08 season. Fungicides applied at specific crop stages were assessed in both trial years, and the results revealed that cyprodinil + fludioxonil applied at PBC resulted in lower BBR severity at harvest relative to other treatments, with this result being statistically significant for the 2008-09 whole block trial. The whole-of-block experiment allowed investigation of spray timing using commerical vineyard practices, which is not possible when using small-plot trials (Crisp *et al*. 2006). The limitation of small-plot experiments has been noted on a number of occasions, especially for assessment of viticultural management practices (Bramley & Lanyon 2003; Bramley *et al.* 2005b; Bishop & Lark 2006; Bramley 2007; Proffit *et al.* 2006; Bramley 2010b; Panten *et al.* 2010; Panten & Bramley 2011).

The whole-of-block experiment highlighted the benefit of using spatially distributed data for studying the epidemiology of viticulturally important diseases (Bramley et al. 2011). This PhD research focused on the association between vine factors and BBR severity in a spatio-temporal context. The results highlighted the fact that there may be more than one epidemic occurring within a vineyard block, which may mean that vines in some areas will develop higher incidence and severity than in other sections. In commercial terms, certain sections may reach winery thresholds for disease severity sooner, resulting in either price penalties or crop rejection. Use of crop variables associated with BBR severity, such as maps representing vine vigour, would at least inform vineyard managers of which areas of a block to focus in for disease assessment in the weeks before harvest.

As with the findings of Valdés-Goméz (2008), vine vigour was a factor contributing to BBR severity in this study; where higher vigour zones (high plant cell density) resulted in more BBR than the lower vigour sections. There was also a weak but statistically significant association between pruning weights and BBR severity in the flowering treatment, while there was no correlation for the PBC treated vines due to

the overall low severity observed. Even though the number of vines assessed for pruning weights was a small subset of the 150 vines, investigating this variable in a more detail across different blocks and varieties might indicate its utility for grower-based assessment of the risk of BBR.

Vine vigour plays a significant role in vegetative and fruit growth, with the size of a canopy affecting vine microclimate and the extent of spray penetration and coverage. Dense canopies can limit spray penetration depending on the spray equipment and calibrated settings. This can result internal bunches and leaves not receiving an even cover of fungicide spray droplets. Vine vigour can be controlled by pruning, water and nutrient applications, and, in some cases, selection of a grape clone. The lower vigour Chardonnay clone G9V7 had lower BBR severity compared to clone I10V1, which is a more vigorous clone, and which had higher severity (Ciarami & Ewart 1995).

Plant cell density (PCD) is a tool which is widely adopted by industry for estimating relative vigour across a vineyard block at *véraison* for performance benchmarking or for informing decisions about selective harvesting (known in the industry as split picking) to improve wine quality. Split picking and fruit grading is used to select fruit parcels and to maximise quality in the premium sector. It allows the grower and winemaker to keep the better parcels of fruit with higher grades separated from the lower quality fruit (lower grades), rather than harvesting the block at once resulting in the fruit ending up in the lower grade market.

The analysis of spatial variation in plant cell density (PCD) showed statistically significant differences between treatments, that varied across the block, *P* values decreased as PCD increased (i.e. differences became more significant). The change of vigour (as estimated by PCD) also correlated with the site position with respect to the slope, since the vines in the upslope area generally had higher vigour than in the lower level area. With this in mind, the previous season's (2007-08) small plot trial was conducted lower level area, where vines had lower vigour, which may have impacted the results, as the results for BBR severity were marginally significant. This finding again highlights issues in using small plot experiments; that is, they do not

necessarily reflect what may actually occur under normal vineyard practices or other sections of the block (Bramley & Lanyon 2003; Crisp *et al*. 2006; Bramley 2010b; Panten & Bramley 2011). They are most useful for testing experimental treatments where the treatment effects are largely unknown (e.g. developmental crop protectants) and where there could be severe crop loss if the experimental treatment was applied across a large proportion of the vineyard block.

Results of the small plot trials suggested that the necrotic tissue pathway was not the main infection pathway for *B. cinerea* at this particular vineyard site, as the trash removal did not have a significant effect on end of season severity, since the incubated trash showed little sign of colonisation by the fungus. Latent infection was probably occurring during the period between flowering and early berry development (EL33) because *B*. *cinerea* was isolated prior to fungicide application. Moreover, the mid-season sprays reduced the amount of BBR expressed at harvest in comparison with the nil treated plots. One hypothesis is that cyprodinil + fludioxonil eradicated some of the latent infections (Zitter and Wilcox 2007b). The results for the whole-of-block experiment were consistent with those of the small-plot trial, since both experiments showed that the PBC spray resulted in a lower BBR severity at harvest than the flowering spray. The spatio-temporal analysis of the flowering treatment in the 2008-09 trial supported the findings of the small plot trial. The analysis suggested that the spatial increase of the BBR severity appeared to be attributed to the expression of latent infection established during the period between flowering and early berry development and/or new infections (via wounds, splits in berries) followed by berry-berry spread within a bunch. There was no evidence to suggest that secondary infection, in which disease spreads from the initial infection site to a new one (i.e. from one bunch to another), was occurring at this site.

Weather plays a crucial role in BBR epidemiology. There were a limited number of rainfall events for the 2008-09 season, but dry weather afterwards reduced the period of higher humidity presumably needed for further colonisation of the berry by the fungus and for fungal sporulation (Zitter and Wilcox 2007a). The rain events that occurred during the final stages of berry development appeared to cause some splitting, although this might have been exacerbated by the 'hen & chicken' disorders

observed. Relative humidity measured within the vine canopy at various locations within the block appeared to be a key driver of the development of BBR; however, RH was generally below the optimal 90% RH (Gubler *et al*. 1987; Nair *et al*. 1988; Thomas *et al*. 1988; English *et al*. 1989; Nicholas *et al*. 1994; Broome *et al.* 1995; Vail *et al*. 1998; Beresford 2007). The low incidence of latent BBR observed at PBC prior to fungicide application during 2008-09, the associated weather conditions and the spatial temporal analysis of the flowering fungicide treatment suggested that secondary infection of *B*. *cinerea* during the growing season was very limited and not the main driver of the progression of BBR.

In summary, application of an effective fungicide at PBC was found to limit latent infection establishment and subsequent BBR development during ripening, whereas the application of the fungicide at flowering only protected the developing fruit for a limited time. Quantification of spatial variation in disease severity and/or factors associated with an elevated BBR risk can provide clues as to practical measures to reduce botrytis risk in targeted sections of a vineyard block, as well as provide options for selective fruit harvesting to manage grape quality. The duplex assay for qPCR will provide a new tool for future studies of BBR epidemiology assuming an appropriate sampling strategy can be implemented and sources of variability such as DNA quality and quantity are reduced. Quantification of *B*. *cinerea* DNA in host tissue over time also needs to take into consideration changes in the quality of host tissue, as well as developing a better understanding of the mechanisms and location of fungal colonisation during infection, latency, and symptom expression.

Recommendations for future research and development

The following recommendations for future research and development are based on the findings of this study and the current literature.

1. Further improve the qPCR technique from DNA extraction to quantification. Use of grape juice or homogenate for extraction of high quality DNA would overcome the laborious and time-consuming process of grinding frozen berries in liquid nitrogen.
2. Quantify changes in host-tissue quality relative to the extent of fungal colonisation and stage of berry development to account for factors that might

be affecting the amount of *B. cinerea* DNA quantified by qPCR. Again, if the qPCR technique can be improved, then it can be tested at more sites and during more seasons to improve knowledge about the spatio-temporal progression of BBR in relation to factors that influence disease epidemiology.

3. Study further the sampling strategy required to obtain a representative sample of grape berries from a vineyard block for qPCR and other quantitative detection methods. The use of grape juice or homogenate, whereby whole bunches are used, might reduce the variance associated with sampling single berries; however, a sufficient sample size is still required.
4. Develop spectroscopy methods further, especially near-infrared spectroscopy used in the wine industry to measure other quality parameters (Cozzolino and Dambergs 2010). Sampling issues might be overcome if a mobile device for 'on-the-go' sensing can be used to scan many bunches quickly (Bramley *et al*. 2011c).
5. When developed, apply the rapid, reliable, and quantitative method for assessing the levels of *B. cinerea* in the vineyard and then identify the threshold level that constitutes a problem for commercial winemaking according to grape variety and wine style (Steel *et al*. 2013).
6. Pruning weights are measured routinely in many vineyards. Explore the use of pruning weight as a practical tool to assess spatial variation in the risk and expression of BBR in both cool and warm climates.
7. Investigate key infection pathways for *B. cinerea* in other Tasmanian vineyards. These pathways might be verified by conducting inoculations of marked strains of *B. cinerea* at key crop stages. It may be possible to use *nit* mutants to track colonisation from the early stages of fruit development up until harvest (Beever and Parkes 2003). By using *nit* mutants, this would enable the tracking of the colonisation of the *B. cinerea* from a known inoculating starting time rather than solely relying on natural populations to become established in the fruit. Zitter & Wilcox (2006) completed a study using potted grapevines to track the colonisation of *B. cinerea* under a variety of environmental conditions. Even though the strain has been selectively mutated, it does not affect the colonisation capabilities of the fungus (Beever & Parkes 2003). The fungus can be isolated at multiple stages to confirm that it is in fact the strain used to the initial inoculation, via subculturing.

8. Investigate further the role of bunch trash (floral remnants) as a source of *B. cinerea* inoculum for different grape varieties grown in Tasmanian vineyards.
9. Develop a software tool to allow ready access to the complex geo-statistical analyses required for future implementation of whole-of-block experimentation.
10. Link the spatially-distributed results of whole-of-block experimentation to precision-viticulture technology so that crop inputs can be varied across a vineyard block according the level of biotic or abiotic stress.

References

Acevedo-Opazo C, Tisseyre B, Guillaume S, Ojeda H (2008) The potential of high spatial resolution information to define within-vineyard zones related to vine water status. *Precision Agriculture* **9**, 285-302.

Agnew RH, Mundy D, Balasubramaniam R (2004) Effects of spraying strategies based on monitored disease risk on grape disease control and fungicide usage in Marlborough. *New Zealand Plant Protection* **57**, 30-36.

Agrios GN (1997) Plant Pathology, 4th edn. Academic Press: San Diego, California, USA.

Allan W (2003) Winegrape assessment in the vineyard and at the winery. Winegrape Growers' Council of Australia and the Winemakers' Federation of Australia: Adelaide, Australia. Project no WFA01/01.

Anonymous (2011a) Tasmania Wine- 2011 in review. Wine Tasmania, Hobart Tasmania.

Anonymous (2011b) Wine Tasmania, 2011 Tasmanian vintage report. Wine Tasmania, Hobart, Tasmania.

Asher M, Cowe IA, Thomas CE, C CD (1982) A rapid method of counting spores of fungal pathogens by infrared analysis. *Plant Pathology* **31**, 363-371.

AWRI (2009) Measuring anthocyanins (colour) in red grape berries.

AWRI (2011) Managing Botrytis infected Fruit.

Bailey PT, Ferguson KL, McMahon R, Wicks TJ (1997) Transmission of *Botrytis cinerea* by lightbrown apple moth larvae on grapes. *Australian Journal of Grape and Wine Research* **3**, 90-94.

Balasubramaniam R, Edwards WRN, Agnew RH, Scott K (2000) A multifaceted approach to grape Botrytis disease management. Proceedings of the Australian Society for Viticulture and Enology seminar 'Managing Bunch Rots' (Eds C Davies, C Dundon and R Johnstone), Mildura . Australian Society for Vitculture and Enology, Adelaide, 12-18.

Baldwin G (2011) Technology for correcting wine faults. Proceedings of the Australian Society of Viticulture and Oenology seminar 'Making the best out of difficult vintages: managing sub-optimal fruit in the winery' (Ed. P. Petrie), Adelaide. *Australian Society of Viticulture and Oenology Inc*., Adelaide South Australia, 7-10.

Barbetti MJ (1980) Reductions on Bunch Rot in Rhine Riesling grapes from bunch thinning. *Australasian Plant Pathology* **9**, 8-10.

Barry K, Corkrey S, Pham T, Mohammed C (2011) Spectral characterization of necrosis from reflectance of *Eucalyptus globulus* leaves with Mycosphaerella leaf disease or subjected to artificial lesions. *International Journal of Remote Sensing* **32**, 9243-9259.

Batten GD (1998) Plant analysis using near infrared reflectance spectroscopy: the potential and the limitations. *Australian Journal of Experimental Agriculture* **38**, 697-706.

Beever RE, Parkes SL (2003) Use of nitrate non-utilising (Nit) mutants to determine vegetative compatibility in *Botryotinia fuckeliana* (*Botrytis cinerea*). *European Journal of Plant Pathology* **109**, 607-613.

Bell S-J, Henschke P (2005) Implications of nitrogen nutrition for grapes, fermentation and wine. *Australian Journal of Grape and Wine Research* **11**, 242-295.

Bennett JS, Trought MPH, Crevan M (2007) The impact of differences in soil type on vine phenology, growth and fruit composition of Sauvignon blanc in a Marlborough vineyard. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide.

Beresford RM (2007) Inoculum and climatic factors associated with epidemics of grape Botrytis in New Zealand. Proceedings of the 16th Biennial Australasian Plant Pathology Society Conference. Adelaide, South Australia. *Australasian Plant Pathology Society Inc*, 89

Beresford RM (2010) Towards reduced reliance on fungicides for disease control in New Zealand's crop-based industries. *New Zealand Plant Protection* **63**, 138-144.

Beresford RM, Evans KJ, Wood PN, Mundy DC (2006) Disease assessment and epidemic monitoring methodology for bunch rot (*Botrytis cinerea*) in grapevines. *New Zealand Plant Protection* **59**, 1-6.

Beresford RM, Hill GN (2008) Predicting in-season risk of botrytis bunch rot in Australian and New Zealand vineyards. Proceedings of the Austalian Society of Viticulture and Oenology seminar 'Breaking the Mould: a Pest and Disease Update'(Eds: K. DeGaris, M. Krstic, G. McCorkelle, S. McLoughlin), Mildura. *Australian Society of Viticulture and Oenology Inc*., Adelaide, South Australia, 24-28.

Beresford RM, Spink M (1992) A national disease forecasting system for apple black splot (*Venturia inaequalis*) in New Zealand. *Acta horticulturae* **313**, 285-296.

Bernard M, Horne PA, Papacek D, Jacometti MA, Wratten SD, Evans KJ, Herbert KS, Powell KS, Rakimov A, Weppler R, Kourmouzis T, Yen AL (2007) Guidelines for environmentally sustainable winegrape production in Australia: IPM adoption self-assessment guide for growers. *Australian & New Zealand Grapegrower & Winemaker* **518**, 24-35.

Bézier A, Lambert B, Baillieul F (2002) Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. *European Journal of Plant Pathology* **108**, 111-120.

Bishop T, Lark R (2006) The geostatistical analysis of experiments at the landscape-scale. *Geoderma* **133**, 87-106.

Blanchfield AL, Robinson SA, Renzullo LJ, Powell KS (2006) Phylloxera-infested grapevines have reduced chlorophyll and increased photoprotective pigment content-can leaf pigment composition aid pest detection? *Functional Plant Biology* **33**, 507-514.

Boonham N, Glover R, Tomlinson J, Mumford R (2008) Exploiting generic platform technologies for the detection and identification of plant pathogens. *European Journal of Plant Pathology* **121**, 355-363.

Bramley RGV (2005) Understanding varibility in winegrape production systems 2. Within vineyard variation in quality over several vintages. *Australian Journal of Grape and Wine Research* **11**, 33-42.

Bramley RGV (2006) Acquiring an informed sense of place, practical applications of Precision Viticulture. *The Australian and New Zealand Wine Industry Journal* **21**, 26-33.

Bramley RGV (2007) Spatial data and experimentation should be an essential part of optimal grape and wine production. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide.

Bramley RGV (2010a) Geostatistical interpolation is a powerful tool for visualising vineyard variability with scarce, unevenly distributed data. Proceedings of the 14th Australian Wine Industry Technical Conference (Eds R Blair, T Lee and S Pretorius). The Australian Wine Industry Technical Conference Inc.: Adelaide.

Bramley RGV (2010b) Precision Viticulture: managing vineyard variability for improved quality outcomes. In 'Managing wine quality. Vol. Volume 1: Viticulture and wine quality.' Ed. AG Reynolds). Woodhead Publishing: Cambridge, pp. 445-480.

Bramley RGV, Evans KJ, Dunne KJ, Gobbett DL (2011) Spatial variation in response to 'reduced input' spray programs for powdery mildew and botrytis identified through whole-of-block experimentation. *Australian Journal of Grape and Wine Research* **17**, 341-350.

Bramley RGV, Evans K, Gobbett D, Panten K, Scott E (2007) Optimising strategies for control of powdery mildew through whole of block experimentation. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide.

Bramley RGV, Hamilton RP (2004) Understanding varibility in winegrape production systems 1. Within vineyard variation in yield over several vintages. *Australian Journal of Grape and Wine Research* **10**, 32-45.

Bramley RGV, Lanyon D, Panten K (2005b) Whole-of-vineyard experimentation - an improved basis for knowledge generation and decision making. Proceedings of the 5th European Conference on Precision Agriculture. (Ed. JV Stafford). Wageningen Academic Publishers: The Netherlands, 883-890.

Bramley RGV, Lanyon D (2003) Making sense of vineyard variability on Australia. Proceedings of Precision Viticulture IX Congreso latinoamericano de Viticultura y Enologia' Centro de Agricultura de Precisión, (Eds R Ortega and A Esser). Santiago, Chile, 34-54

Bramley RGV, Le Moigne M, Evain S, Ouzman J, Florin L, Fadaili EM, Hinze CJ and Cerovic ZG (2011c) On-the-go sensing of grape berry anthocyanins during commercial harvest: development and prospects. *Australian Journal of Grape and Wine Research* **17**, 316–326.

Bramley RGV, Proffit A, Hinze C, Pearse B, Hamilton R (2005a) Generating benefits from Precision Viticulture through selective harvesting. Proceedings of the 5th European Conference on Precision Agriculture. (Ed. JV Stafford), Wageningen Academic Press: Netherlands, 891-898.

Bramley RGV, Trought M (2010) Characterising vineyard variability in Marlborough, New Zealand. Proceedings of the 14th Australian Wine Industry Technical Conference (Eds R Blair, T Lee and S Pretorius). The Australian Wine Industry Technical Conference Inc.: Adelaide.

Bramley RGV, Trought M, Praat J-P (2011) Vineyard variability in Marlborough, New Zealand: characterising variation in vineyard performance and options for the implementation of Precision Viticulture. *Australian Journal of Grape and Wine Research* **17**, 72-78.

Bramley RGV, Williams S (2001) A protocol for the construction of yield maps from data collected using commercially available grape yield monitors. *Cooperative Research Centre for Viticulture*. Adelaide, South Australia.

Braybrook D (2007) Development of regional IPM best practice. Final report to the GWRDC, Project No. VTS 06/01.

Breuil A-C, Jeandet P, Adrian M, Chopin F, Pirio N, Meunier P, Bessis R (1999) Characterisation of a pterostilbene dehydrodimer produced by laccase of *Botrytis cinerea*. *Phytopathology* **89**, 298-302.

Broome JC, English JT, Marois JJ, Latorre BA, Aviles JC (1995) Development of a infection model for Botrytis Bunch Rot of grapes based on wetness duration and temperature. *Phytopathology* **85**, 97-102.

Brouwer M, Livens B, Hemelrijck WV, Ackerveken GVd, Cammue BPA, Thomma BPHJ (2003) Quantification of disease progression of several microbial pathogens on *Aribidopsis thaliana* using real-time fluorescence PCR. *FEMS Microbiology Letters* **228**, 241-248.

Bruce R, Lamb DW, Runting J, Powell KS (2009) A precision agriculture approach to targeted serveillance for grapevine phylloxera in vineyards. Proceedings of the13th Annual Symposium on Precision Agriculture in Australasia (Eds MG Trotter, EB Garraway and DW Lamb). University of New England, 78.

Brys G, Hubert M, Struyf A (2004) A robust measure of skewness. *Journal of Computational and Graphical Statistics* **13**, 996-1017.

Bulit J, Dubos B (1988) Botrytis bunch rot and blight. In 'compendium of grape diseases.' (Eds RC Pearson and AC Goheen). APS Press, The American Phytopathological Society, 13-15.

Bustin S, A, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publications of quantitative real-time PCR experiments. *Clinical Chemistry Reviews* **55**, 1-11.

Cadle-Davidson L (2008) Monitoring pathogenesis of natural *Botrytis cinerea* infections in developing grape berries. *American Journal of Enology and Viticulture* **59**, 387-395.

Celik M, Kalpulov T, Zutahy Y, Ish-shalom S, Lurie S, Lichter A (2009) Quantitative and qualitative analysis of Botrytis inoculated on table grapes by qPCR and antibodies. *Postharvest Biology and Technology* **52**, 235-239.

Chandrasene G, Brune AE, Rutherford RS, Dharmawardena N (2003) Detection of phytoplasmas associated with grassy shoot and white leaf diseases of sugarcane in Sri Lanka using FTA™ papers. *Sugar Tech* **5**, 237-241.

Chardonnet C, L'Hyvernay A, Doneche B (1997) Effect of calcium treatment prior to *Botrytis cinerea* infection on the changes in pectic composition of grape berry. *Physiological and Molecular Plant Pathology* **50**, 213-218.

Cirami RM, Ewart AJW (1995) Clonal selection, evaluation and multiplication in Australia. Proceedings of the International Symposium on clonal selection (Ed. JM Rantz), 52-59.

Cole M (2004) Botrytis- its place in the vineyard Part 1- History. *The Australia & New Zealand Grapegrower & Winemaker* **480**, 22-24.

Cole M (2005) Management of Botrytis bunch rot in grapevines by nutritional applications. *Australian Viticulture*, **September/October**, 31-32.

Cole M (2005) Demonstration of best practice Botrytis control strategies on a vineyard in the alpine Valleys wine region of greater Victoria. GWRDC Report No. RT 02/40-4.

Cole M, Whiting J, Hood V, Laukart N, Blackshaw P (2004) Botrytis management checklist. Monash University.

Cole M, Whitmore S, Wiechel T, Ford C, Hornell J, Leeton P (2004) Botrytis and its place in the vineyard. Part 2: Distribution of Botrytis in the vineyard. *The Australia & New Zealand Grapegrower & Winemaker* **482**, 15-18.

Cole M, Wiechel T (2004) Demonstration of best practice Botrytis control strategies on a vineyard in the Yarra Valley regions of greater Victoria. GRWDC Project No. RT 02/38-3.

Coley-Smith JR, Verhoeff K, Jarvis WR (Eds) (1980) The biology of Botrytis. Academic Presss: London, Great Britain

Comménil P, Brunet L, Aduran J-C (1997) The development of the grape berry cuticle in relation to susceptibility to bunch rot disease. *Journal of Experimental Botany* **48**, 1599-1607.

Cook DWM (2002) Effect of formulated yeast in suppressing the liberation of *Botrytis cinerea* conidia. *Plant Disease* **86**, 1265-1270.

Cook DWM, Elmer PAG, Hill RA (2002) Quantitative inoculation with dry conidia of *Botrytis cinerea*. *Australasian Plant Pathology* **31**, 217-222.

Coolong TW, Walcott RR, William RM (2008) A quantitative real-time polymerase chain reaction assay for *Botrytis aclada* in onion bulb tissue. *HortScience* **43**, 408-413.

Coombe BG (1960) Relationship of growth and development to changes in sugards, auxins, and gibberellins in fruit of seeded and seedless varieties of *Vitis vinifera*. *Plant Physiology* **35**, 241- 250.

Coombe BG (1995) Adoption of a system for identifying grapevine growth stages. *Australian Journal of Grape and Wine Research* **1**, 104- 110.

Coombe BG, Dry PR (Eds) (1992a) 'Practices.' Viticulture Volume 2 Winetitles: Adelaide.

Coombe BG, Dry PR (Eds) (1992b) Resources. Viticulture Volume 1. Winetitles: Adelaide.

Coombe BG, Hale CR (1973) The hormone content of ripening grape berries and the effects of growth substance treatments. *Plant Physiology* **51**, 629-634.

Cortell JM, Halbleib M, Gallagher AV, Righetti T, L, Kennedy JA (2005) Influence of vine vigor on grapes (*Vitis vinifera* L. Cv. Pinot Noir) and wine proanthocyanidins.

Cozzolino D, Cynkar WU, Janik LJ, Francis IL, Gishen M, Dambergs RG (2003) Advances in infrared (IR) spectroscopy at AWRI: novel applications for grape and wine analysis. *The Australian Wine Research Institute, Urrbrae, SA*. **Technical Review No. 145**, 11-14, 35-37.

Cozzolino D, Blazquez I, Smith P, Bartowsky EJ, Cynkar WU, Janik LJ, Dambergs RG, Gishen M (2007a) The use of visible and near infrared spectroscopy combined with chemometrics to analyse spectral patterns related to different Saccharomyces species and strains in red wine. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide.

Cozzolino D, Cynkar WU, Dambergs RG, Janik LJ, Gishen M (2007b) Near infrared spectroscopy in the Australian grape & wine industry. *CRVC Technology Application Note* **April**, 1-20.

Cozzolino D, Cynkar W, Dambergs RG, Shah N, Smith PA (2010a) Rapid and objective analysis of wine. Proceedings of the 14th Australian Wine Industry Technical Conference (Eds R Blair, T Lee and S Pretorius). The Australian Wine Industry Technical Conference Inc.: Adelaide.

Cozzolino D, Cynkar WU, Janik LJ, Francis IL, Gishen M, Dambergs RG (2003) Advances in infrared (IR) spectroscopy at AWRI: novel applications for grape and wine analysis. *The Australian Wine Research Institute, Urrbrae, SA*. **Technical Review No. 145**, 11-14, 35-37.

Cozzolino D, Cynkar W, Shah N, Smith PA (2010b) Rapid and objective analysis of grape composition. Proceedings of the 14th Australian Wine Industry Technical Conference (Eds R Blair, T Lee and S Pretorius). The Australian Wine Industry Technical Conference Inc.: Adelaide, 374.

Cozzolino, D. and Dambergs, R.G. (2010) Instrumental analysis of grape, must and wine. In: Managing wine quality (Ed. A.G. Reynolds). Woodhead Publishing Limited: Oxford) 134–161.

Cozzolino D, Elser MB, Dambergs RG, Cynkar WU, Boehm DR, Francis IL, Gishen M (2004) Prediction of colour and pH in grapes using a diode array spectrophometer (400-1100 nm). *Journal of Near Infrared Spectroscopy* **12**, 105-111.

Cozzolino D, Smyth HE, Sefton MA, Herderich MJ, Gishen M, Dambergs RG, Janik LJ, Cynkar WU (2007c) Preliminary study on the use of near infrared spectroscopy as a rapid tool to predict volatile aroma compounds in Australian Riesling wine. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide.

Crisp P, Wicks TJ, Bruer N, Scott ES (2006) An evaulation of biological and abiotic controls for grapevine powdery mildew. 2. Vineyard trials. *Australian Journal of Grape and Wine Research* **12**, 203-211.

CropLife - Australia Fungicide Resistance Management Review Group (2011) Fungicide resistance managment strategy. CropLift Australia Ltd

Culbert J, Gishen M, Day C, Oemcke D (2008) Taking the sting out of spoilage. *Australian & New Zealand Wine Industry Journal* **23**, 47-50.

Dambergs RG, Stummer B, Bevin C, Lim A, Cozzolino D, Gishen M, Scott ES (2007) Rapid analysis of powdery mildew in grapes: an industry trial. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide.

de Kock, P.J., and Holz, G. (1994) Application of fungicides against postharvest *Botrytis cinerea* bunch rot of table grapes in the Western Cape. *South African Journal of Enology and Viticulture* **15**, 33–40.

Delaherche A, Claisse O, Lonvaud-Funel A (2004) Detection and quantification of *Brettanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. *Journal of Applied Microbiology* **97**, 910-915.

Delalieux S, van Aardt J, Keulemans W, Schrevens E, Coppin P (2007) Detection of biotic stress (*Venturia inaequalis*) inapple tress using hyperspectral data: Non-paraetric statistical approaches and physiological implications. *European Journal of Agronomy* **27**, 130-143.

Dewey FM, Elbeler SE, Adams DO, Noble AC, Meyer UM (2000) Quantification of *Botrytis* in grape juice determined by a monoclonal antibody-based immunoassay. *American Journal of Enology and Viticulture* **51**, 276-282.

Dewey FM, Hill M, DeScenzo R (2008) Quantification of Botrytis and laccase in winegrapes. *American Journal of Enology and Viticulture* **59**, 47-54.

Dewey F, Meyer U, Danks C (2005) Rapid immunoassays for stable Botrytis antigens in pre- and postsymptomatic grape berries, grape juice and wines. Proceedings of the American Society of Enology and Viticulture 56th Annual Meeting, 302A-303A

Dewey FM, Steel CC, Gurr SJ (2013) Lateral-flow devinces to rapidly determine levels of stable Botrytis antigens in table and dessert wines. *American Journal of Enology & Viticulture* **64**, 291-295.

Dewey FM, Yohalem D (2007) Detection, quantification and immunolocalisation of Botrytis species. In Botrytis, Pathology and Control (Ed. Y Elad). Kluwer Academic Publishers: Netherlands. 181-194.

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

Dobrowski SZ, Ustin SL, Wolpert JA (2003) Grapevine dormant pruning weight prediction using remotely sensed data. *Australian Journal of Grape and Wine Research* **9**, 177-182.

Dorak MT (2010) Glossary of Real-time PCR Terms [Online). Available: http://www.dorak.info/genetics/glosrt.html (Accessed 21st January, 2010).

Dorak MT (2011) Real-Time PCR [Online]. Available: http://www.dorak.info/genetics/realtime.html (Accessed 10th October, 2011).

Dowell FE, Ram MS, Seitz LM (1999) Predicting scab, vomitoxin and ergosterol in single wheat kernels using near-infrared spectroscopy. *Cereal Chemistry* **76**, 573-576.

Dry I, Thomas M (2003) Modification of grape cluster architecture for control of fungal diseases. Grape and Wine Research and Development Corporation**,** Cooperative Research Centre for Viticulture, No. CRV 99/14d, Glen Osmond, South Australia.

Dubos, B., and Roudet, J. (2000) First results of the research network on vine grey rot epidemiology in France. Proceedings of the 12th International Botrytis Symposium, Reims, France, 44.

Dugan FM, Lupien SL, Grove GG (2002) Incidence, aggressiveness and *in planta* interactions of *Botrytis cinerea* and other filamentous fungi quiescent in grape berries and dormant buds in central washington state. *Journal of Phytopathology* **150**, 375-381.

Dumeau F, Murat ML, Marquette B, Daviaud F (2004) The effect of tannin addition on laccase activity of *Botrytis cinerea* during fermentation. *The Australia & New Zealand Grapegrower & Winemaker*, 78-86.

Edwards J, Riches D, Evans K, Beresford R, Hill G, Wood P, Mundy D (2009) The need for a risk-based approach to botrytis management. *The Australian & New Zealand Grapegrower & Winemaker* **Annual Technical Issue,** 6-9.

Eilenberg J, Hajek A, Lomer C (2001) Suggestions for unifying the terminology in biological control. *BioControl* **46**, 387-400.

Elad Y, Shtienberg D (1994) Effect of compost water extracts on grey mould (*Botrytis cinerea*). *Crop Protection* **13**, 109-114.

Ellison P, Ash G, McDonald (1998a) An expert system for the management of Botrytis cinerea in Australian vineyards I. Development. *Agricultural Systems* **56**, 185-207

Ellison P, Ash G, McDonald (1998b) An expert system for the management of Botrytis cinerea in Australian vineyards. II. Validation. *Agricultural Systems* **56,** 209-224.

Elmer PAG, Hoyte SM, Vanneste JL, Reglinski T, Wood PN, Parry FJ (2005) Biological control of fruit pathogens. *New Zealand Plant Protection* **58**, 47-54.

Elmer PAG, Michailides TJ (2004) Epidemiology of *Botrytis cinerea* in orchard and vine crops. In 'Botrytis: Biology, Pathology and Control' (Ed. Y Elad). Kluwer Academic Publishers: Netherlands, 243-272.

Elmer PAG, Reglinski T (2006) Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology* **55**, 155-177.

Emmett B, Rozario S, Hawtin J, Buchanan G, Warren M, Mebalds M, Laukart N, Wiechel T, Strawhorn J, Whitmore S, Smith M, Braybrook D, Tabatabai S, Hepworth G, Reynolds J, Wicks T, Bailey P, McMahon R, Hall B, Campbell K, Ferguson K, Hoffmann A, Bennet D, Robinson M, Scott M, Thomson L, Wood R, Brown A, Glenn D, Nair T, Villagran K, Cole M (2005) Strategic management of botrytis bunch rot and lightbrown apple moth on grapevines. Final report to GWRDC, Project No. DAV 95/1.

Emmett B, Ashley R, Bell S, Cameron S, Clarke K, Clake S, Dambergs B, Francis L, Gale K, Hill A, Ailsa H, Kazi B, Leong S-l, Markides A, Markovic V, Mebalds M, Nancarrow N, Pitt J, Reynolds J, Scott E, Stummer B, Varelis P, Waters E, Wicks T, Zanker T (2004) Fungal contaminates and their impact on wine quality. Final report to GWRDC, Project No. CRV 99/23.

Emmett RW, Clingeleffer PR, Wicks TJ, Nair NG, Hall B, Hart KM, Clarke K, Somers T (1994) Influence of canopy architecture on disease development. Proceedings of the Australian Society of Viticulture Oenology Seminar 'Canopy management'. (Ed. P Hayes), *Australian Society of Viticulture and Oenology Inc*., Adelaide, South Australia. 22-23, 36

English JT, Thomas CS, Marois JJ, Gubler WD (1989) Microclimates of grapevine canopies associated with leaf removal and control of botrytis bunch rot. *Phytopathology* **79**, 395-401.

Envirologix (2007) QuickStix Kit for Botrytis in Wine Grape Juice. Enivirologix

Essling M, Longbottom M (2013) Agrochemicals registered for use in Australian viticulture 11/12. The Australian Wine Research Institute: Glen Osmond, South Australia.

Evans KJ (2008) Overview of R&D for managing botrytis bunch rot in Australia. Proceedings of the Austalian Society of Viticulture and Oenology seminar 'Breaking the mould: a pest and disease update'(Eds: K. DeGaris, M. Krstic, G. McCorkelle, S. McLoughlin), Mildura. *Australian Society of Viticulture and Oenology Inc*., Adelaide, South Australia, 4–15.

Evans KJ (2010) Botrytis Management. Tasmanian Institute of Agricultural Researchm University of Tasmania. GWRDC Factsheet.

Evans KJ (2011) Assessing and managing disease-affected fruit in the vineyard: the Australian experience. Proceedings of the Australian Society of Viticulture and Oenology seminar 'Making the best out of difficult vintages: managing sub-optimal fruit in the winery' (Ed. P. Petrie), Adelaide. *Australian Society of Viticulture and Oenology Inc*., Adelaide South Australia, 11-19.

Evans KJ, Beresford RM, Hill GN (2010a) Quantitative methods for studies of botrytis bunch rot in grapevines. In 'Effective management of botrytis bunch rot for cool climate'. Final report to GWRDC, Project No UT0601, 15-34.

Evans KJ, Dunne KJ, Riches D, Edwards J, Beresford RM, Hill GN (2010b) Effective management of botrytis bunch rot for cool climate viticulture. Final Report to GWRDC, Project No. UT 06/01.

Evans KJ, Gadoury D (2008) What is 80% capfall? *Australian & New Zealand Grapegrower & Winemaker* **36th Annual Technical Issue**, 16-20.

Evans KJ, Palmer AK, Metcalf DA (2013) Effect of aerated compost tea on grapevine powdery mildew, botrytis bunch rot and microbial abundance on leaves. *European Journal of Plant Pathology* **135**, 661-673.

Evans KJ, Whisson DL, Scott, EL (1996) An experimental system for characterizing isolates of *Uncinula necator*. *Mycological Resarch* **100**, 675-680.

Fermaud M, Valdés-Goméz H, Calonnec A, Roudet J, Gary C (2007) Interdependent effects of (micro) climate and vegetative growth on grey mould incidence in grapevine. Proceedings of the 14th International Botrytis Symposium ', Cape Town, South Africa.

Gabler MF, Smilanick JL, Mansour M, Ramming DW, Mackey BE (2003) Correlations of morphological, anatomical, and chemical features of grape berries with resistance to *Botrytis cinerea*. *Phytopathology* **93**, 1263-1273.

Gachon C, Mingam A, Charrier B (2004) Real-time PCR: what relevance to plant studies? *Journal of Experimental Botany* **55**, 1445-1454.

Gao X, Jackson TA, Lambert KN, Li S, Hartman GL, Niblack TL (2004) Dection and quantification of *Fusarium solani*, f.sp. *glycines*, in soybean roots and with Real-time Quantitative Polymerase Chain Reaction. *Plant Disease* **88**, 1372-1380.

Gindreau E, Walling E, Lonvaud-Funel (2001) Direct polymerase chain reaction detection of ropy *Pediococcus damnosus* strains in wine. *Journal of Applied Microbiology* **90**, 535-542.

Gishen M, Dambergs RG, Cozzolino D (2005) Grape and wine analysis - enhancing the power of spectroscopy with chemometrics. A review of some applications in the Australian wine industry. *Australian Journal of Grape and Wine Research* **11**, 296-305.

Gishen M, Cozzolino D, Dambergs RG (2010) The analysis of grapes, wine and other alcoholic beverages by infrared spectroscopy. In 'Applications of vibrational spectroscopy in food science, Analysis of food, drink and related Materials'. Vol. II. (Eds ECY Li-Chan, PR Griffiths and JM Chalmers). John Wiley & Sons Litd: West Sussex, United Kingdom, 539-556.

Gladstones J (1992) 'Viticulture and Environment'. Winetitles: Adelaide.

Glick BR, Pasternak JJ (1998) Molecular Biotechnology: Principles and Applications of Recombinant DNA, 2nd Edition edn. ASM Press: Washington, D.C.

Godden P (2000) Bunch rots- understanding the winemaker's dilemma. Proceedings of the Australian Society for Viticulture and Enology seminar 'Managing Bunch Rots' (Eds C Davies, C Dundon and R Johnstone), Mildura . Australian Society for Vitculture and Enology, Adelaide, 52-54.

Godden P (2003) Rapid fermenting start inhbits taint. *National Grape Growers* (April), 35.

Goto M, Honda E, Ogura A, Notomo A, Hanaki KI (2009) Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy napthol blue. *BioTechniques* **46**, 167-172.

Grund E, Darissa O, Adam G (2010) Application of FTA® Cards to sample microbial plant pathogens for PCR ad RT-PCR. *Journal of Phytopathology* **158**, 750-757.

Gubler WD, Marois JJ, Bledsoe AM, Bettiga LJ (1987) Control of Botrytis Bunch Rot of grape with canopy management. *Plant Disease* **71**, 599-601.

Gubler WD, Rademacher MR, Vasquez, Thomas C (1999) Control of powdery mildew using the UC Davis powdery mildew risk index. In Plant pathology online, www.apsnet.org (acessed 21st April 2014).

Guilbaud-Oulton S (2000) Getting it all together. Proceedings of the Australian Society for Viticulture and Enology seminar 'Managing Bunch Rots' (Eds C Davies, C Dundon and R Johnstone), Mildura . Australian Society for Vitculture and Enology, Adelaide, 55-57.

Haboudane D, Miller JR, Pattey E, Zarco-Tejada P, J, Strachan IB (2004) Hyperspectral vegetation indices and novel algorithms for predicting green LAI of crop canopies: Modeling and validation in the context of precision agriculture. *Remote Sensing of Environment* **90**, 337-352.

Hale CR (1968) Growth and senescence of grape berry. *Australian Journal of Agricultural Research* **19**, 936-945.

Hall B, Emmett B (2000) Bunch rots: what, how and when. Proceedings of the Australian Society for Viticulture and Enology seminar 'Managing Bunch Rots' (Eds C Davies, C Dundon and R Johnstone), Mildura . Australian Society for Vitculture and Enology, Adelaide, 7-11.

Hall A, Lamb DW, Holzapfel BP, Louis JP (2011) Within-season temporal variation in correlations between vineyard canopy and winegrape composition and yield. *Precision Agriculture* **12**, 103-117.

Harris JM, Kriedemann PE, Possingham JV (1968) Anatomical aspects of grape berry development. *Vitis* **7**, 106-108.

Hayden K, Ivors K, Wilkison C, Garbelotto M (2006) Taqman® chemistry for *Phytophthora ramorum* detection and quantification, with a comparison of diagnostic methods. *Phytopathology* **96**, 846-854.

Hed B, Ngugi HK, Travis JW (2009) Relationship between cluster compactness and bunch rot in Vignoles grapes. *Plant Disease* **93**, 1195-1201.

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genome Research* **6**, 986-994.

Hill G, Stellwaag-Kittler F, Huth G, Schlösser E (1981) Resistance of grapes in different developmental stages to *Botrytis cinerea*. *Phytopathology* **102**, 328-338.

Holz G, Gütschow M, Coertze S, Calitz F (2003) Occurrence of *Botrytis cinerea* and subsequent disease expression at different positions on leaves and bunches of grape. *Plant Disease* **87**, 351-358.

Howell G (2011) Botrytis cinerea: Australian vintage 2011. Australian & New Zealand Grapegrower & Winemaker **570**, 67-68.

Iland P, Bruer N, Edwards G, Weeks S, Wilkes E (2004) Chemical analysis of grapes and wine: techniques and concepts. Winetitles: Adelaide, Australia.

Jacobi J, Kühbauch W (2005) Site-specific identification of fungal infection and nitrogen deficiency in wheat crop using remote sensing. Proceedings of the 5th European Conference on Precision Agriculture (Ed. JV Stafford). Wageningen Academic Press: Netherlands, 73-80.

Jacometti MA, Wratten SD, Walter M (2007) Management of understorey to reduce the primary inoculum of *Botrytis cinerea*: Enhacing ecosystem services in vineyards. *Biological Control* **40**, 57-64.

Jacometti MA, Wratten SD, Walter M (2010) Review: alternatives to synthetic fungicides for *Botrytis cinerea* management in vineyards. *Australian Journal of Grape and Wine Research* **16**, 154-172.

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo N, Jubin C, Vezzi A, Legeai F, Hugueney P, Dasilva C, Horner D, Mica E, Jublot D, Poulain J, Bruyère C, Billault A, Segurens B, Gouyvenoux M, Ugarte E, Cattonaro F, Anthouard V, Vico V, Del Fabbro C, Alaux M D, Gaspero G, Dumas V, Felice N, Paillard S, Juman I, Moroldo M, Scalabrin S, Canaguier A L, Clainche I, Malacrida G, Durand E, Pesole G, Laucou V CP, Merdinoglu D, , Delledonne M, Pezzotti M, Lecharny A, Scarpelli C, Artiguenave F, Pè ME, Valle G,

Morgante M, Caboche M, Adam-Blondon AF, Weissenbach J, Quétier F, P; W, Characterization (French- Italian Public Consortium for Grapevine Genome Characterization). (2007) The grapevine genome sequence suggests ancestral hexaploidization in majorangiosperm phyla. *Nature* **449**, 463-467.

Jaspers M.V, Seyb A.M, Trought M.C.T, Balasubramaniam R (2013) Overwintering grapevine debris as an important source of *Botrytis cinerea* inoculum. *Plant Pathology* **62**, 130-138.

Javier Rodriguez-Rajo F, Jato V, Fernández-González M, Jesus Aria M (2010) The use of aerobiological methods for forecasting Botrytis spore concentrations in a vineyard. *Grana* **49**, 56-65.

Jeger MJ, Viljanen-Rollinson (2001) The use of the area under the disease-progress curve (AUDPC) to assess quantitative disease resistance in crop cultivars. *Theoretical and Applied Genetics* **102**, 32-40.

Keller M, Viret O, Cole M (2003) *Botrytis cinerea* infection in grape flowers: defense reaction, latency, and disease expression. *Phytopathology* **93**, 316-322.

Kennedy AM (2002) An Australian case study: introduction of new quality measures and technologies in the viticultural industry. Proceedings of the 11th Australian Wine Industry Technical Conference. Australian Wine Industry Technical Conference Inc:. Adelaide, 199-205.

Kernaghan G, Reeleder RD, Hoke SMT (2007) Quantification of *Cylindrocarpon destructans* f. sp. *panacis* in soils by real-time PCR. *Plant Pathology* **56**, 508-516.

Kessel GJT, De Haas B, Van Der Werf W, Köhl J (2002) Competitive substrate colonisation by *Botrytis cinerea* and *Ulocladium atrum* in relation to biological control of *B. cinerea* in cyclamen. *Mycological Research* **106**, 716-728.

Kingston C (2001) Disease management under IWP - what does this mean? The Australia & New Zealand Grapegrower & Winemaker (formerly New Zealand Grapegrower & Winemaker) **446,** 47-50.

Korelev N, Mamiev M, Zahavi T, Elad Y (2011) Screening of *Botrytis cinerea* isolates from vineyards in Israel for resistance to fungicides. *European Journal of Plant Pathology* **129**, 591-608.

Kos G, Krska R, Lohninger H, Griffiths PR (2004) A comparative study of mid-infrared diffue reflection (DR) and attenuated total reflection (ATR) spectroscopy for the detection of fungal infection on RWA2-corn. *Analytical and bioanlytical chemistry* **378**, 159-166.

Kretschmer M, Kassemeyer H, Hahn M (2007) Age-dependent grey mould susceptibility and tissue-specific defence gene activation of grapevine berry skins after infection by *Botrytis cinerea*. *Journal of Phytopathogy* **155**, 258-263.

Krstic M, Moulds G, Panagiotopoulos B, West S (Eds) (2003) Growing Quality Grapes to Winery Specifications. Winetitles: Adelaide.

Kulakiotu EK, Thanassoulopoulos CC, Sfakiotakis EM (2004) Biological control of *Botrytis cinerea* by volatiles of 'Isabella' grapes. *Journal of Phytopathology* **94**, 924-931.

Lanyon D, Bramley RGV (2004) Ameliorating soil constraints to the performance of established vineyards. Final Report to the Grape and Wine Research Development Corporation: Adelaide.

Lark R, Bellamy P, Rawlins BG (2006) Spatio-temporal variability of some metal concentrations in the soil of eastern England and implications for soil monitoring. *Geoderma* **133**, 363-379.

Larsolle A, Hamid Muhammed H (2005) Measuring crop status using multivariate analysis of hyperspectral field reflectance with application to disease severity and plant density. Proceedings of the 5th European Conference on Precision Agriculture (Ed. JV Stafford). Wageningen Academic Press: Netherlands, 217-225.

Leyronas C, Nicot P (2013) Monitoring viable airborne inoculum of *Botrytis cinerea* in the south-east of France over 3 years: relation with climatic parameters and the origin of air masses. *Aerobiologia* **29**, 291-299.

Lin H, Walker M (1997) Extracting DNA from cambium tissue for analysis of grape rootstocks. *HortScience* **32**, 1264-1266.

Livak KJ, Flood SJA, Marmaro J, Giusti W, Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *Genome Research* **4**, 357-362.

Loconsole G, Saponari M, Savino V (2010) Development of real-time PCR based assays for simultaneous and improved detection of citrus viruses. *European Journal of Plant Pathology* **128**, 251-259.

Lorenz DH, Eichhorn KW, Bleiholder H, Klose R, Meier U, Weber E (1995) Growth stages of the grapevine. *Australian Journal of Grape and Wine Research* **1**, 100-110.

Lorrain IK, Jourdes M, Pasquier G, Fermaud M, Gény L, Rey P, Doneche B, Teissedre P-L (2012) Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quaility of Bordeaux grapes, musts and wines for two consecuative vintages. *Australian Journal of Grape and Wine Research* **18**, 215-226.

Luo Y, Michailides T (2001) Factors affecting latent infection of prune fruit by *Monilinia fructicola*. *Phytopathology* **91**, 864-872.

Ma Z, Michailides TJ (2005) Genetic Structure of *Botrytis cinerea* populations from different host plants in California. *Plant Disease* **89**, 1083-1089.

MacGregor A, Riches D, Gaskin R, Manktelow D (2006) Better disease control using adjuvants. GRWDC Project No. DNR 02/04.

Maeda H, Kokeguchi S, Fujimoto C, Tanimoto I, Yoshizumi W, Nishimura F, Takashiba S (2005) Detection of peridontal pathogen *Porphyromonas gingivalis* by loop-mediated isothermal amplification method. *FEMS Immunol Medical Microbiolgy* **43**, 233-239.

Magnin-Robert M, Trotel-Aziz P, Quantinet D, Biagianti S, Aziz A (2007) Biological control of *Botrytis cinerea* by selected grapevine-associated bacteria and stimulation of chitinase and β-1,3 glucanase activities under field conditions. *European Journal of Plant Pathology* **118**, 43-57.

Marois JJ, Kenyon LH, Lamison CD, Smith CJ (1994) A rapid immunoassay method for quantifying mold in harvested grapes. *American Journal of Enology and Viticulture* **45**, 300-304.

Martin L (2001) Studies for the Prevention of Red Wine Browning- development of a molecular control the the Copper Enzyme laccase. Final project to GWRDC, Project No. UF 96/1.

McCartney H, Foster S, Fraaije B, Ward E (2003) Molecular diagnostics for fungal plant pathogens. *Pest Managment Science* **59**, 129-142.

McClellan WD, Hewitt W (1973) Early Botrytis rot of grapes: Time of infection and latency of *Botrytis cinerea* Pers. in *Vitis vinifera* L. *Phytopathology* **63**, 1151-1157.

Mehli L, Kjellsen T, Dewey F, Hietala A (2005) A case study from the interaction of strawberry and *Botrytis cinerea* highlights the benefits of comonitoring both partners at genomic and mRNA level. *New Phytologist* **168**, 465-474.

Mertely JC, MacKenzie SJ, Legard DE (2002) Timing of fungicide applications for *Botrytis cinerea* based on development stage of strawberry slowers and fruit. *Plant Disease* **86**, 1019-1024.

Meyer U, Spotts R, Dewey F (2000) Detection and quantification of *Botrytis cinerea* by ELISA in pear stems during cold storage. *Plant Disease* **84**, 1099-1103.

Michailides TJ, Elmer PAG (2000) Botrytis gray mold of kiwifruit Caused by *Botrytis cinerea* in the United States and New Zealand. *Plant Disease* **84**, 208-223.

Minasny B, McBratney AB, Whelan BM (2005) VESPER version 1.62. Australian Centre for Precision Agriculture, University of Sydney: Sydney, NSW.

Minerdi D, Moretti M, Li Y, Gaggero L, Garibaldi A, Gullino ML (2008) Conventional PCR and real-time quantitative PCR dection of *Phytophthora cryptogea* on *Gerbera jamesonii*. *European Journal of Plant Pathology* **122**, 227-237.
Mohapatra NK, Mukherjee AK, Rao AVS, Nayak P (2008) Disease progress curves in the rice blast pathosystem compared with the logistic and gompertz models. *ARPN Journal of Agricultural and Biological Science* **3**, 28-37.

Mori Y, Nagamine K, Tomita N, Notomi T (2001) Dection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Bioch Biophys Res Commun* **289**, 150-154.

Mori Y, Kitao M, Tomita N, Notomi T (2004) Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical Biophys Methods* **59**, 145-157.

Moyano C, Alfonso C, Gallego J, Raposo R, Melgarejo P (2003) Comparison of RAPD and AFLP marker analysis as a means to study the genetic structure of *Botrytis cinerea* populations. *European Journal of Plant Pathology* **109**, 515-522.

Mullins MG, Bouquet A, Williams LE (1992) Biology of the Grapevine. Cambridge University Press: Cambridge, United Kingdom.

Mundy DC, Beresford RM (2002) Managing bunch rot more effectively through understanding the epidemiology of botrytis. *The Australia & New Zealand Grapegrower & Winemaker,* **Annual Technical Issue**, 15-17.

Mundy DC, Beresford RM (2007) Susceptibility of grapes to *Botrytis cinerea* in relation to berry Nitrogen and sugar concentration. *New Zealand Plant Protection* **60**, 123-127.

Murashige T, Skoog K (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.

Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular Cell Probes* **16**, 223-229.

Nagamine K, Watanabe K, Ohtsuka K, Hase T, Notomi T (2001) Loop-mediated isothermal amplification reaction using a nondenatured template. *Clinical Chemistry* **47**, 1742-1743.

Nair NG, Emmett RW, Parker FE (1988) Some factors predisposing grape berries to infection by *Botrytis cinerea*. *New Zealand Journal of Experimental Agriculture* **16**, 257-263.

Nair NG, Guilbaud-Oulton S, Barchia I, Emmett R (1995) Significance of carry over inoculum, flower infection and latency on the incidence of *Botrytis cinerea* in berries of grapevines at harvest in New South Wales. *Australian Journal of Experimental Agriculture* **35**, 1177-1180.

Nair NG, Nadtotchei A (1987) Sclerotia of Botrytis as a source of primary inoculum for bunch rot of grapes in New South Wales, Australia. *Journal of Phytopathology* **119**, 42-51.

Nair NG, Parker FE (1985) Midseason bunch rot of grapes: an unusual disease phenomemnon in the Hunter Valley, Australia. *Plant Pathology* **34**, 302-305.

Nazarenko I, Pires R, Lowe B, Obaidy M, Rashtchian A (2002) Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. *Nucleic Acids Research* **30**, 2089-2195.

Nicholas P, Magarey P, Wachtel M (Eds) (1994) Diseases and pests, Grape Production Series. Winetitials: Adelaide, South Australia.

Notomi T, Okayama H, Masubuchi H, Yonemawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* **28**, e63.

Obanor FO, Walter M, Waipara NW, Cernusko R (2002) Rapid method for the detection and quantification of *Botrytis cinerea* in plant tissues. *New Zealand Plant Protection* **55**, 150-153.

Obanor FO, Williamson K, Mundy DC, Wood PN, Walter M (2004) Optimisation of PTA-ELISA Dection and quantification of *Botrytis cinerea* infections in grapes. *New Zealand Plant Protection* **57**, 130-137.

Oliveira AC, Vallim MA, Semighini CP, Araújo WL, Goldman GH, Machado MA (2002) Quantification of *Xylella fastidiosa* from citrus trees by Real-time Polymerase Chain Reaction assay. *Phytopathology* **92**, 1048-1054.

Pak H, Wood P (1995) Resistance of Botrytis to fungicides in Hawkes Bay vineyards. Proceedings of the 48th New Zealand Plant Protection Conference', pp. 27-30.

Pal K, McSpadden Gardener B (2006) Biological Control of Plant Pathogens. *The Plant Health Instructor*, 1-25.

Panten K, Bramley R (2007) Exploring canopy management as a tool to improve wine quality using whole of block experimentation. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide.

Panten K, Bramley R (2011) Viticulture experiments using whole blocks: evaluation of three floor management options. *Australian Journal of Grape and Wine Research* **17**, 136-146.

Panten K, Bramley R, Lark R, Bishop T (2010) Enhancing the value of field experimentation through whole-of-block designs. *Precision Agriculture* **11**, 198-213.

Pashkoulov D, Giannetti I, Benvenuto E, De Matinis D (2002) Biochemical characterization of polygalacturonases from five different isolates of *Botrytis cinerea*. *Mycological Research* **106**, 827-831.

Pearson RC (1984) botrytis bunch rot and blight. *New York State Agricultural Experimental Station*.

Peason T, Wicklow D, Marghirang E, Xie F, Dowell F (2001) Detecting aflatoxin in single corn kernels by transmittance and reflectance spectroscopy. *Transactions of the American Society of Agricultural Engineers* **44**, 1247-1254.

Peynaud E (1984) 'Knowing and Making Wine'. John Wiley & Sons, Inc.: Paris.

Pezet R, Viret O, Perret C, Tabacchi R (2003) Latency of *Botrytis cinerea* Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, Respectively Susceptible and Resistant to Grey Mould. *Journal of Phytopathology* **151**, 208-214.

Proffit T, Bramley R, Lamb D, Winter E (2006) Precision Viticulture, A new era in vineyard management and wine production. Winetitles Pty Ltd: Adelaide, South Australia.

Rabosto X, Carrau M, Paz A, Boido E, Dellascassa E, Carrau FM (2006) Grapes and vineyard soils as sources of microorganisms for biological control of *Botrytis cinerea*. *American Journal of Enology & Viticulture* **57**, 332-338.

Rankine B (1998) Making Good Wine: A manual of winemaking practice for Australia and New Zealand. Pan Macmillan Australia Ptd Limited: Sydney.

Reglinski T, Elmer PAG, Taylor JT, Parry FJ, Marsden R, Wood PN (2005) Suppression of Botrytis bunch rot in Chardonnay grapevines by induction of host resistance and fungal antagonism. *Australasian Plant Pathology* **34**, 481-488.

Reglinski T, Elmer PAG, J.T T, Wood PN, Hoyte SM (2010) Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan. *Plant Pathology* **59**, 882-890.

Reglinski T, Kingston C (2001) Biological control- the next generation of pest control agents. *The Australia & New Zealand Grapegrower & Winemaker*, 106-109.

Ricker RW, Marois JJ, Dlott JW, Bostock RM, Morrison JC (1991) Immunodetection and quantification of *Botrytis cinerea* on harvested winegrapes. *Phytopathology* **81**, 404-411.

Rigotti S, Gindro K, Richter H, Viret O (2002) Characterization of molecular markers for specific and sensitive detection of *Botrytis cinerea* Pers.: Fr. in strawberry (*Fragaria* × *ananassa* Duch.) using PCR. *Microbiology Letters* **209**, 169-174.

Riley L (2008) Practical management of bunch rots in high risk environments. Proceedings of the Austalian Society of Viticulture and Oenology seminar 'Breaking the Mould: a Pest and Disease Update' (Eds: K. DeGaris, M. Krstic, G. McCorkelle, S. McLoughlin), Mildura. *Australian Society of Viticulture and Oenology Inc*., Adelaide, 19-23.

Rogiers S, Greer L, Weckert M, Steel C, Schmidtke L, Holzapfel B (2003) Agricultural spray adjuvants: effects on grape berry wax, berry microflora and subsequent botrytis infection. *The Australia & New Zealand Grapegrower & Winemaker*, 19-21.

Rogiers S, Whitelaw-Weckert M, Radovanonic-Tesic M, Greer L, White RG, Steel C (2005) Effects of spray adjuvants on grape (*Vitis vinifera*) berry microflora, epicuticular wax and susceptibility to infection by *Botrytis cinerea*. *Australasian Plant Pathology* **34**, 221-228.

Rozario S, Emmett B, Strawhorn J, Whitmore S, Hawtin J (2005) Effects of the removal of floral trash from bunches on the development of botrytis bunch rot of grapes. In ‘'Strategic management of Botrytis bunch rot and lightbrown apple moth on grapevines’. Final report to GWRDC, Project Number DAV 95/1. Department of Primary Industries, 75-77.

Salinas J, Schots A (1994) Monoclonal antibodies-based immunofluorescence test for detection of conidia of *Botrytis cinerea* on cut flowers. *Phytopathology* **84**, 351-356.

Sanderson D (2012a) Climate Data for Tasmania. (Wine Tasmania: Hobart)

Sanderson D (2012b) Tasmania - The Reign of Terrior. (Wine Tasmania: Hobart)

Sanzani SM, Schena L; De Cirro V, Ippolito A (2012) Early detection of *Botrytis cinerea* latent infections as a tool to improve postharvest qualtiy of table grapes. *Postharvest biology and technology* **68**, 64-71.

Savazzini F, Martinelli L (2006) DNA analysis in wines: development of methods for enhanced extraction and real-time polymerase chain reaction quantification. *Anal Chim*. *Acta* **563**, 274-282.

Schena L, Nigro F, Ippolito A, Gallitelli D (2004) Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology* **110**, 893-908.

Schiler AMC, Gillett JM, Sysak RW, Wise JC (2002) Evaluation of environmentally friendly products for control of fungal diseases of grapes. Proceedings of the 10th International Conference on Cultivarion Technique and Phytopathological Problems in Organic Fruit-Growing and Viticulture', Foko: Weinsberg, Germany, pp. 163-167.

Schmidt K (2007) *Thrips obscuratus* in New Zealand vineyards: its biology and effects on *Botrytis cinerea* infection. PhD Thesis, Lincoln University, Christchurch.

Schmidt K, Teulon DAJ, Wratten SD, Jaspers M (2007) *Thrips obscuratus*, as a vector of *Botrytis cinerea* to cause grape flower infection. Proceedings of the 14th International Botrytis Symposium', Cape Town, South Africa.

Schmittgen T.D (2001) Real-time Quantitative PCR. *Methods* **25**, 383-385.

Scholefield P, Morison J (2010) Assessment of economic cost of endemic pests & diseases on the Australian grape & wine industry. Grape & Wine Research & Development Corporation, No. GWR 08/04.

Scott ES, Dambergs RG, Stummer B (2010) Fungal conteminants in the vineyard and wine quality. In 'Managing wine quality, Volume 1: viticulture and wine quality (Ed. AG Reynolds). Woodhead Publishing LImited/ CRC Press, 481-509

Segarra G, Casanova E, Borrero C, Avilés M, Trillas I (2007) The suppressive effects of composts used as growth media against *Botrytis cinerea* in cucumber plants. *European Journal of Plant Pathology* **117**, 393-402.

Selma MV, Martinez-Culebras PV, Aznar R (2008) Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. *International Journal of Food Microbiology* **122**, 126-134.

Shavrukov YN, Dry IB, Thomas MR (2003) Inflorescence and bunch architecture development in *Viti vinifera* L. *Australian Journal of Grape and Wine Research* **10**, 116-124.
Smart R, Robinson M (1991) Sunlight into Wine, A handbook for winegrape canopy managment. Winetitles, Adelaide.

Smart RE (1992) Canopy Management. In Viticulture, Practices. Volume 2. (Eds BG Coombe and PR Dry).Winetitles, Adelaide.

Smith CJ, Osborn AM (2008) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbiology. *FEMS Microbiology and Ecology* **67**, 6-20.

Smith M (2011) Heat treatment stars in rescue of laccase wine. *Australian & New Zealand Grapegrower & Winemaker* **575**, 41-43.

Steel CC, Blackman JW, Schmidtke LM (2013) Grapevine bunch rots: impacts on wine composition, quality, and potential procedures for the removal of wine faults. Journal of Agricultural and Food Chemistry **61**, 5189-5206

Stoll M, Schultz HR, Baecker G, Berkelmann-Loehnertz B (2008) Early pathogen detection under different water status and the assessment of spray application in vineayrds through the use of thermal imagery. *Precision Agriculture* **9**, 407- 417.

Stummer B, Zanker T, Harvey PR, Scott E (2006) Detection and quantification of *Erysiphe necator* DNA in wine grapes and resultant must and juice. *Mycological Research* 110, 1184-1192.

Suarez M, Walsh K, Boonham N, O'Neill T, Pearson S, Barker I (2005) Development of real-time PCR (Taqman®) assays for the detection and quantification of *Botrytis cinerea* in planta. *Plant Physiology and Biochemistry* 43, 890-899.

Symons GM, Davies C, Shavrukov Y, Dry IB, Reid JB, Thomas M (2006) Grapes on steroids. Brassinosteroids are involved in grape berry ripening. *Plant Physiology* 140, 1-9.

Tartachnyk I, Rademacher I, Kühbauch W (2005) Discrimination between nitrogen deficiency and fungal infection of winter wheat by laser induced fluorescence. Proceedings of the 5th European Conference on Precision Agriculture (Ed. JV Stafford). Wageningen Academic Press: Netherlands, 65-72.

Thomas CS, Marois JJ, English JT (1988) The effects of wind Speed, temperature, and relative humidity on development of aerial mycelium and conidia of *Botrytis cinerea* on grape. *Phytopathology* **78**, 260-265.

Thompson JR, Latorre BA (1999) Characterisation of *Botrytis cinerea* from table grapes in Chile using RAPD-PCR. *Plant Disease* **83**, 1090-1094.

Tessonnière H, Vidal S, Barnavon L, Alexandre H, Remize F (2009) Design and performance of a real-time PCR assay for sensitive and reliable direct quantification of *Brettanomyces* in wine. *International Journal of Food Microbiology* **129**, 237-243.

Tomlinson JA, Dickinson MJ, Boonham N (2010a) Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. Letters in Applied Microbiology **51**, 650-657.

Tomlinson JA, Dickinson MJ, Boonham N (2010b) Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. Phyopathology 100, 143-149.

Tortora GJ, Funke BR, Case CL (2001) 'Microbiology an Introduction.' 7th edn. (Benjamin Cummings: San Francisco, USA)

Trought MCT, Dix R, Mills T, Greven M, Agnew R, Mauk JL, Praat J-P (2008) The impact of differences in soil texture within a vineyard on vine vigour, vine earliness and juice composition. *Journal international des Sciences de la Vigne et du Vin* **42**, 67-72.

Tyerman SD, De Beil R, Sullivan W, Cynkar WU, Cozzolino D, Dambergs RG NIR spectroscopy to detect water status of grapevines. Proceedings of the 13th Australian Wine Industry Techncial Conference (Eds R Blair, P Williams and I Pretorius). Australian Wine Industry Technical Conference Inc:. Adelaide

Ugliano M, Kwiatkowski M, Waters E (2010) VIS-NIR spectroscope to predict selected chemical parameters in unopened bottles of Sauvignon Blanc wines. Proceedings of the 14th Australian Wine Industry Technical Conference (Eds R Blair, T Lee and S Pretorius). The Australian Wine Industry Technical Conference Inc.: Adelaide, 385.

Valasek MA, Repa JJ (2005) The power of real-time PCR. *Advances in Physiology Education* **29**, 151-159.

Valdés-Goméz H, Fermaud M, Roudet J, Calonnec A, Gary C (2008) Grey mould incidence is reduced on grapevines with lower vegetative and reproductive growth. *Crop Protection* **27**, 1174-1186.

Vail ME, Wolpert JA, Gubler WD, Rademacher MR (1998) Effect of cluster tightness on Botrytis Bunch Rot in six Chardonnay clones. *Plant Disease* **82**, 107-109.

Valsesia G, Gobbin D, Patocchi A, Vecchione A, Pertot ICG (2005) Development of a high-throughput method for quantification of *Plasmopara viticola* DNA in grapevine leaves by means of quantitative real-time Polymerase Chain Reaction. *Phytopathology* **95**, 672-678.

Van Kan JAL (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science* **11**, 247-253.

van Maanen A, Xu X-M (2003) Modelling plant disease epidemics. *European Jounral of Plant Patholgy* **109**, 669-2003.

van Tongeren SP, Degener JE, Harmsen HJM (2011) Comparison of three rapid and easy bacterial DNA extraction methods for use with quantitative real-time PCR. *European Journal of Clinical Microbiology and Infectious Diseases* **30**, 1053-1061.

Varma A, Padh H, Shrivastava (2007) Plant genomic DNA isolation: An art or a science. *Biotechology Journal* **2**, 386-292.

Verhoeff K (1980) The infection Process and Host-Pathogen Interactions. In 'The Biology of *Botrytis* (Eds JR Coley-Smith, K Verhoeff and WR Jarvis). Academic Press: London, 153-180.

Versari A, Parpinello G, Mattioli A, Galassi S (2008) Determination of grape quality at harvest using fourier-transform mid-infrared spectroscopy and multivariate Analysis. *American Journal of Enology and Viticulture* **59,** 317-322.

Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL Lorito M (2008) Trichoderma-plant-pathogen interactions. *Soil Biology & Biochemistry* **40**, 1-10.

Ward E, Foster SJ, Fraaije BA, McCartney HA (2004) Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology* **145**, 1-16.

Warren M, Laukart N, Mebalds M, Strawhorn J, Whitmore S, Rozario S, Emmett B (2005) Sources of Botrytis inoculum in vineyards and their management. In 'Strategic management of Botrytis bunch rot and lightbrown apple moth on grapevines'. Final report to GWRDC, Project Number DAV 95/1. Department of Primary Industries 44-51.

Webster R, Oliver MA (2007) 'Geostatistics for Environmental Scientists. Wiley & Sons: Chichester.

Whatman FTA Nucleic Acid collection, storage and purification.

Wicks T (2002) Improving fungicide control of Botrytis Bunch Rot. Grape and Wine Research and Development Corporation**,** South Australian Research and Development Institution, No. SAR 01/02.

Wicks T, Hall B (2004) Botrytis fungicides- more research needed on spray timing. *The Australia & New Zealand Grapegrower & Winemaker* **476**, 47- 48.

Wilcox WF (2002) Controlling botrytis: a perspective from the eastern USA. *The Australia & New Zealand Grapegrower & Winemaker* **464,** 22-27.

Wilcox W, Zitter S, Lakso A (2006) Effects of atmospheric & soil moisture and fungicide physical mode of action on the development of Botrytis Bunch Rot. Cornell University, Geneva.

Wilhelm J, Pingould A (2003) Real-Time Polymerase Chain Reaction. *ChemBioChem* **4**, 1120-1128.

Williamson B, Tudzynski B, Tudzynski P, Van Kan JA (2007) Pathogen profile: *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology* **8**, 561-580.

Wine Tasmania (2014) Map of Tasmanian wine regions http://winetasmania.com.au/resources/downloads/Tasmanian_Wine_Map.jpg (Acessed on the 13th April 2014).

Winkler AJ, Cook JA, Kliewer WM, Lider LA (1974) General Viticulture, 2nd edn. University of California Press: Berkeley, Los Angeles.

Winter E, Nicol G (1998) Protecting against *Botrytis cinerea* infection in grapes. *The Australian Grapegrower & Winemaker* **November**, 51-52.

Wolf TK, Baudoin ABAM, Martinez-Ochoa N (1997) Effect of floral debris removal from fruit clusters on botrytis bunch rot of Chardonnay grapes. *Vitis* **36**, 27-33.

Zheng L, Campbell M, Murphy J, Lam S, Xu J-R (2000) The *BMP1* Gene is essential for pathogenicity in the gray mold fungus *Botrytis cinerea*. *Molecular Plant-Microbe Interactions* **13**, 724-732.

Zitter SM (2005) The biology and control of Botrytis Bunch Rot (*Botrytis cinerea*) in grapevines: ontogenic, physical and cultural factors affecting initiation and spread of the disease. PhD Dissertation Thesis, Cornell University.

Zitter SM, Wilcox WF (2006) Temporal, physical, and cultural factors affecting the initiation, activation, and spread of Botrytis infections. Proceedings of the Sixth International Cool Climate Symposium for Viticulture & Oenology. Christchurch, New Zealand.

Zitter SM, Wilcox WF (2007a) Environmetal and nutritional factors influencing the activation and spread of quiescent infections of *Botrytis cinerea* in grapes. Proceedings of the 14th International Botrytis Symposium.Cape Town, South Africa, 44.

Zitter SM, Wilcox WF (2007b) Physical modes of action of fungicides used for control of botrytis bunch rot of grapes. Proceedings of the 14th International Botrytis Symposium. Cape Town, South Africa, 129.

Appendix A

Media, Solutions and Buffers

Media

Lactic Acid Potato Dextrose Agar (LPDA) (1 L volume)

Chemical	Company	Amount
Potato Dextrose Agar	Oxoid Australia Pty Ltd, Adelaide	19.5 g
Technical 3 Agar		5 g
75 % Lactic Acid	-	1.4 mL

Preparation of the LPDA media was based on that of Shurtleff & Averre (1997). Agar was dissolved in dH_20 and sterilised at 121 °C for 15 min. The agar was then left to cool to approximately 50 °C prior to adding the lactic acid (Shurtleff & Averre, 1997).

Tissue Culture Media

Chemical	Manufacturer	Amount	Concentration
MS salts	Sigma Aldrich	2.2 g	
Sucrose		7.5 g	1.5%
Technical 3 Agar	Oxoid Australia Pty Ltd, Adelaide	5 g	1 %

Dissolve the MS salts (Murashige and Skoog salts and vitamins) and sucrose in dH_20; adjust pH to 5.7 using NaOH, make volume up to 500 mL. Agar was then dissolved in the solution with the aid of a hotplate. The media was then poured in tissue culture tubes and sterilised at 121 °C for 15 min. Agar was then left to cool until set.

DNA Extraction Buffers

Buffer A (1 L)

Chemical	Manufacturer	Amount (g)	Concentration (mM)
Sorbitol (FW 182.2)	Sigma Aldrich	63.77	350
Trizma base (FW 121)	Astral Scientific	12.1	100
EDTA (ethylenediaminetetraacetic acid)		1.68	6
Sodium bisulphite	Sigma Aldrich	3.81	37
Sodium borate ($Na_2B_4O_7.10H_2O$)		76.27	200

Adjusted to pH of 7.5 using 32 % hydrochloric acid. All components were dissolved in sterile deionised water to a final volume of 1 L.

Buffer B: Nuclei Lysis Buffer (200 mL)

Chemical	Manufacturer	Amount	Concentration (M)
Tris (1M, pH 7.5) [a]	Astral Scientific	40 mL	0.2
EDTA (0.5 M, pH 8.0) [a]		20 mL	0.1
NaCl (5M) [a]		80 mL	2.0
CTAB (hexadecyl trimethylammonium bromide)		4g	0.055
dH_20		60 mL	

[a] Prepared solutions prior to mixing for buffers

Buffer C: Sarcosyl (100 mL)

Chemical	Manufacturer	Amount	Concentration (%)
N-laurosarcosine	Sigma Aldrich	5 g	5

Dissolved in 100 mL sterile dH_20.

Electrophoresis Buffers

20 X Lithium borate buffer

Chemical	Manufacturer	Amount	Concentration
Lithium hydroxide monohydrate	Sigma Aldrich	8.392g	
Boric Acid	Sigma Aldrich	36g	

Dissolved in 950 mL of dH_20 and adjusted to a pH of 8.2. Volume was then made up to a total of 1L.

To run gels stock solution was diluted to 1 X solution with the final concentration of lithium borate to be about 10 mM.

Appendix B
Equations

Equation B1

Heat degree days (HDD) = cumulative value for each month from September - April
n of days/ month where temperature is above 10 °C

Where: -
HDD (month) = n days (Temp >10 °C) × mean month min × (mean max temp – 10)
(Gladstone 1998)
Note: Data presented was that of Sanderson 2012

Equation B2

Efficiency (E) (%) = $(10^{(-1/slope)}-1) \times 100$
(Bustin *et al*. 2009)

Equation B3

Log [DNA Standard] = LOG (10^6 STD)

Equation B4

LOG [*B*. *cinerea* DNA] = (Ct – intercept)/slope

Equation B5

DNA = $10^{LOG\,[B.\,cinerea\ DNA]}$/ (20 10^5)

(Cadle-Davidson 2008)

Equation B6

Logit severity = ln ((% severity + 0.1)/ (100.1 -% severity))
(Beresford *et al*. 2006)

Equation B7:

Back-transformed severity (%) = 100.2 / e (-*logit severity*) - 0.1
(Beresford *et al*. 2006)

Equation B8.1

$V_{cone} = 1/3\pi r^2 l$

Where: -

r = ½ width of bunch at widest point

l = length of bunch

Equation B8.2

$$\text{Per cent of openness (\%)} = \frac{V_{cone} - V_{actual}}{V_{cone}} \times 100 \; \% \; V_{cone}$$

Where: -

V_{actual} = weight (g) of displaced water where density of water = 1

V_{cone} = refer to Equation A.8.1

(Shavrukov *et al*. 2003)

Equation B9:

TA of juice= 0.75 X titrate volume (mL).

(Iland *et al*. 2004)

Equation B10

Transformed Value = Arcsine (√% DNA/100) × (180/π)

Appendix C

Data Tables from Chapter Two

Table C 1: Cycle Threshold (Ct) values from qPCR, which used 2, 3 and 4 µL of DNA standard solution per reaction. Ct values shown are for the detection of both *B. cinerea* and the control/dilutant *V. vinifera* DNA. Samples were tested in duplicate; standard error (SE) is shown in brackets.

B. cinerea DNA Concentration (ng/µL)	Volume of DNA solution (µL)			*V. vinifera* DNA Concentration (ng/µL)	Volume of DNA solution (µL)		
	2 µL (SE)	3 µL (SE)	4 µL (SE)		2 µL (SE)	3 µL (SE)	4 µL (SE)
5	23.09 (-)	22.71 (-)	22.42 (-)	**0**	-	-	-
2.5	24.34 (0.16)	23.71 (0.04)	23.43 (0.14)	**0.1**	29.53 (0.50)	30.26 (0.03)	29.96 (0.24)
0.5	26.14 (0.11)	25.31 (0.03)	25.31 (0.03)	**0.18**	28.45 (0.01)	28.72 (0.28)	29.36 (0.40)
0.1	28.21 (0.26)	28.00(0.12)	27.76 (0.08)	**0.196**	27.86 (0.43)	28.03 (0.08)	28.33 (0.10)
0.02	31.09 (0.04)	30.57 (0.04)	30.18 (0.08)	**0.1992**	27.64 (0.53)	27.78 (0.32)	28.51 (0.16)
0.004	33.99 (0.06)	33.50 (0.12)	33.03 (0.22)	**0.19984**	27.50 (0.51)	27.83 (0.40)	28.44 (0.14)
0.0008	37.45 (1.16)	36.49 (0.40)	36.29 (0.10)	**0.199968**	29.05 (2.29)	28.43 (0.76)	28.12 (0.15)
0.00016	38.76 (-)	39.04 (-)	39.70 (0.29)	**0.1999936**	26.88 (0.01)	27.28 (0.62)	27.95 (0.16)
0	-	-	-	**0.2**	27.34 (-)	26.93 (-)	28.74 (-)

Table C2: Cycle Threshold Values (Ct) for the dilution series using a 2.5 µL volume of DNA solution per reaction and the 0.2 ng/µL *V. vinifera* stock as dilutant. Ct values are for both *B. cinerea* (BC) and *V. vinifera* (Vv). Dilution series were tested in duplicate, standard error (SE) is shown in brackets.

Standard	DNA Amount (ng/ reaction)		Total DNA	Ct BC (SE)	Ct Vv (SE)
	B. cinerea	*V. vinifera*			
Botrytis	12.5	-	12.5	19.99 (-)	-
1	6.25	0.25	6.5	20.63 (0.28)	32.78 (0.54)
2	1.25	0.45	1.7	22.00 (0.11)	28.26 (0.07)
3	0.25	0.49	0.74	24.97 (0.08)	27.44 (0.08)
4	0.05	0.498	0.548	27.04 (0.09)	27.67 (0.30)
5	0.01	0.4996	0.5096	29.77 (0.66)	27.54 (0.32)
6	0.002	0.49992	0.50192	32.18 (0.29)	27.85 (0.19)
7	0.0004	0.499984	0.500384	37.95 (0.03)	27.77 (0.11)
Grape	-	0.5	0.5	-	27.82 (-)

Table C3: Cycle Threshold Values (Ct) quantification of *V. vinifera* DNA using either a 5 ng/µL or 0.2 ng/µL stock solution of *V. vinifera* cv Chardonnay DNA. The latter was prepared by diluting the 5 ng/µL stock solution by a factor of 25. Standard error (SE) is also shown, samples were tested in duplicate.

V. vinifera stock (5 ng/µL)		*V. vinifera* stock (0.2 ng/µL)	
Amount of DNA ng/ reaction	Ct Value (SE)	Amount of DNA ng/ reaction	Ct Value (SE)
12.5	0 (-)	0.5	26.53 (-)
6.25	0 (-)	0.25	27.53 (0.44)
1.25	38.96 (-)	0.05	29.31 (0.12)
0.25	27.07 (0.51)	0.01	31.59 (0.72)
0.05	29.05 (0.25)	0.002	33.55 (0.66)
0.01	31.74 (0.16)	0.0004	34.84 (0.10)
0.002	33.48 (1.81)	0.00008	36.52 (-)
0.0004	35.49 (0.18)	0.000016	36.27 (1.43)

Table C4: Averaged cycle threshold values for dilution series of *B. cinerea* DNA diluted in water or *V. vinifera* DNA using a 0.2 ng/µL stock solution (see Table 2.8). Dilution series was tested in triplicate, standard error (SE) shown in brackets.

Amount of *B. cinerea* DNA (ng/ reaction)	Cycle Threshold Value (Ct)	
	B. cinerea diluted in H_2O (SE)	*B. cinerea* diluted in *V. vinifera* cv. Chardonnay DNA (SE)
6.25	22.80 (0.17)	23.05 (0.07)
1.25	23.83 (0.14)	24.13 (0.15)
0.25	26.41 (0.21)	27.00(0.14)
0.05	29.20 (0.27)	31.65 (1.29)
0.01	31.71 (0.11)	33.00 (0.12)
0.002	34.47 (0.10)	34.47 (0.18)
0.0004	36.98 (0.57)	34.85 (0.04)

Table C5: Cycle threshold values for the optimised simplex and duplex assays. *B. cinerea* DNA was diluted in *V. vinifera* cv Chardonnay DNA. Standard error (SE) is also shown in parentheses.

Amount of *B. cinerea* DNA (ng/reaction)	Ct Value *B. cinerea* DNA		Amount of *V. vinifera* DNA (ng/reaction)	Ct Value for *V. vinifera* DNA	
	Simplex	Duplex		Simplex	Duplex
12.5	22.86 (0)	22.77 (0)	0	0	0
6.25	23.05 (0.07)	22.93 (0.02)	0.25	27.77 (0.12)	28.15 (0.38)
1.25	24.13 (0.15)	23.94 (0.07)	0.45	26.17 (0.12)	26.69 (0.47)
0.25	27.00 (0.14)	27.18 (0.32)	0.49	26.27 (0.01)	26.38 (0.01)
0.05	31.65 (1.29)	30.13 (0.18)	0.498	26.99 (0.16)	26.47 (0.06)
0.01	33.00 (0.12)	34.24 (0)	0.4996	27.27 (0.15)	26.60 (0.15)
0.002	34.47 (0.18)	35.84 (1.73)	0.49992	27.05 (0.02)	26.63 (0.31)
0.0004	34.85 (0.04)	38.70 (0)	0.499984	26.59 (0.05)	26.76 (0.04)
0	0	0	0.5	26.42 (0.36)	26.46 (0.03)

Appendix D

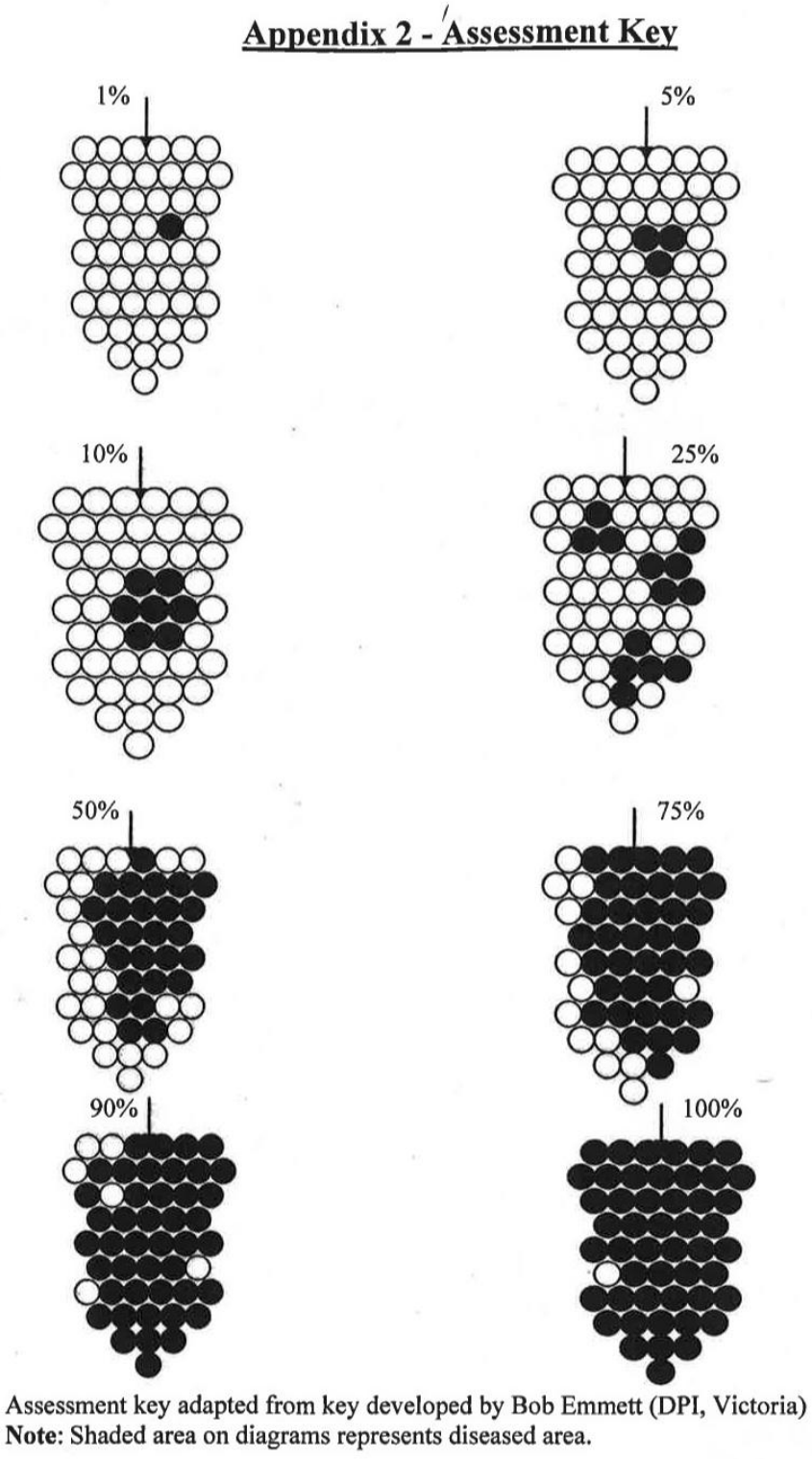

Figure D 1: Assessment key from which visual scoring for disease and physical damage was based on. Key is from the Botrytis Management Check list (Cole *et al.* 2004)

Appendix E
Results from Chapter Four

Table E 1: Mean percentage incidence of *B. cinerea* in berries following ONFIT berries after 9 days of incubation. Berries were from either the nil or pea size fungicide treatments with either trash (Yes) or without trash removal (No). Factorial ANOVA was completed to determine treatment differences (total residual df = 23). The P values from the analysis are as follows: - fungicide *P* = 0.222; trash removal *P* = 0.385; interaction *P* = 0.091. Analysis was conducted using logit-transformed values (in parenthesis).

Growth Stage	Fungicide	Trash Removal		Mean
		Yes	No	
Nil		3.44 (- 3.39)	2.50 (- 5.13)	2.97 (- 4.26)
Pea	cyprodinil+fludioxonil	0.63 (- 5.37)	1.77 (- 4.79)	1.20 (- 5.08)
	Mean	2.04 (- 4.38)	2.14 (- 4.96)	

Table E 2: Mean BBR severity of bunches prior to harvest (7th April 2008). Categories included pink brown plump berries characteristic of BBR (% PBP); shrivel pink brown berries characteristic of old BBR infection (% Shri); total BBR (pink brown plump + shrivelled); sporulating berries within the bunch (% Spor); and percentage incidence.

Trt	Fungicide	Trash	% PBP	% Shri	% Total BBR	% Spor	% Incidence BBR
1	Nil	+	1.54	3.29	4.81	0.40	75
2		-	1.17	2.42	3.58	0.36	77.38
3	Pea	+	0.29	2.97	3.26	0.10	79.76
4		-	0.32	2.85	3.17	0.08	67.26
5	PBC	+	0.08	2.36	2.44	0.02	58.33
6		-	0.56	2.81	3.39	0.20	63.99
7	Flint	+	0.35	2.06	2.41	0.11	57.74
8		-	0.20	2.65	2.85	0.03	67.86

Table E3: Mean percentage incidence of BBR on the 2nd April 2008. Factorial Analysis of Variance (ANOVA) was completed (residual df = 35) to determine if there were any significant effects on the treatments. The calculated *P* values are as followed:- growth stage/ fungicide treatment *P* = 0.719; trash removal *P* = 0.278; interaction *P* = 0.137.

Growth Stage	Fungicide	Trash Removal		Mean
		Yes	No	
Nil		27.98	33.33	30.65
Pea	cyprodinil+fludioxonil	32.44	24.4	28.57
PBC	cyprodinil+fludioxonil	29.17	34.52	31.85
Ver + 3wks	trifloxystrobin	22.62	32.74	27.68
	Mean	28.13	31.25	

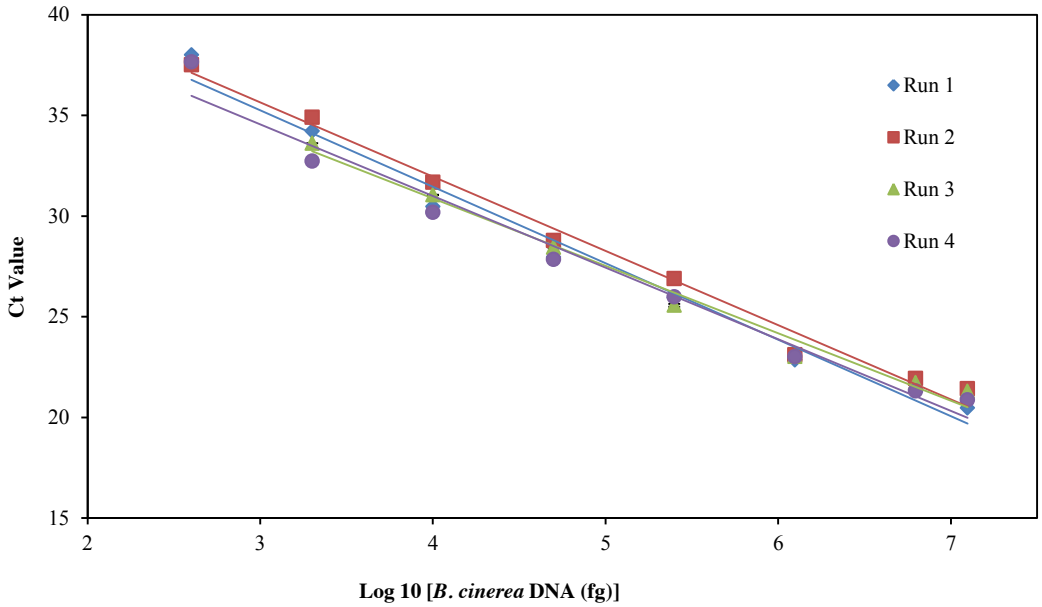

Figure E1: Standards for qPCR Linear Regression Lines. The mean Ct value is shown for each standard in each run. Dilution series was run in duplicate.

Table E4: The slope, intercept and calculated reaction efficiency for the standards for each of the qPCR runs.

Run	Slope	Intercept	R^2	Reaction Efficiency (%)
Run 1	- 3.800	46.658	0.98	83.29
Run 2	- 3.687	46.702	0.99	86.75
Run 3	- 3.347	44.266	0.99	98.97
Run 4	- 3.557	45.226	0.98	91.03

Appendix F
Tables for Chapter Five

Table F 1: Mean severity scores (%) for both flowering and PBC treatments. Bunches were assessed for disease severity (Total Botrytis), area of sporulating *B. cinerea* and other bunch rot damage. Incidence of BBR is shown for each of the assessment dates. BBR severity is broken down into Pink Turgid berries (new infections), shrivelled pink berries (berries started to collapse- older botrytis infection), Pink split berries (infected by botrytis) Standard error of the mean is shown (SE).

Disease & Damage Type	10th March 2008				18th March 2008				24th March 2008				3rd April 2008			
	Flowering		PBC		Flowering		PBC		Flowering		PBC		Flowering		PBC	
	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE	%	SE
BBR Incidence [a]	175	-	263	-	179	-	218	-	816	-	568	-	780	-	507	-
Pink Turgid Berries	0.085	0.020	0.135	0.027	0.118	0.028	0.118	0.028	3.292	0.318	1.323	0.156	1.107	0.241	0.407	0.094
Shrivelled Pink	0.102	0.017	0.194	0.029	0.177	0.027	0.236	0.031	0.323	0.042	0.312	0.036	2.364	0.176	1.095	0.103
Pink Total	0.187	0.028	0.329	0.042	0.295	0.047	0.353	0.045	3.615	0.321	1.635	0.153	3.471	0.314	1.502	0.173
Pink Split	0.002	0.001	0.069	0.021	0.001	0.001	0.00	-	0.003	0.002	0.006	0.002	0.022	0.022	0.012	0.011
BBR severity (turgid + shrivelled)	0.189	0.028	0.398	0.048	0.295	0.047	0.353	0.045	3.618	0.321	1.641	0.153	3.493	0.314	1.514	0.173
Sporulation severity	0.056	0.013	0.091	0.014	0.165	0.028	0.161	0.023	2.739	0.291	1.033	0.123	2.726	0.296	0.989	0.150
Other rots	0.209	0.062	0.00	-	12.88	0.799	12.29	0.559		-		-	16.87	0.558	19.38	0.583
Splitting	24.30	1.194	23.48	0.985	-	-	-	-		-		-	-	-	-	-

[a] Total number of bunches with BBR

[b] Other rots included sour, Penicillium which were not consistent with BBR (identified via comparison with figures and prior knowledge)

Table F 2: Mean percentage of pink turgid berries symptomatic of BBR on the 3rd April 2009. An unbalanced ANOVA was completed using logit transformed vauels hwoen in parenthesis (total residual df = 290). The calculatedted P values from the ANOVA are as followes with the calculated least significant difference (lsd) in parenthesis: - vigour *P* = 0.011 (0.4694); fungicide treatment *P* = <0.001 (0.2925); interaction *P* = 0.407. .

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	0.45 (- 6.02)	0.25 (- 6.253)	0.35 (- 6.135)
Low - Medium	1.24 (- 5.48)	0.50 (- 6.047)	0.88 (- 5.763)
Medium	0.32 (- 6.06)	0.15 (- 6.351)	0.23 (- 6.204)
Medium - High	1.94 (- 5.10)	0.33 (- 6.056)	1.14 (- 5.578) a
High	1.21 (- 5.54)	0.85 (- 5.774)	1.03 (- 5.655) a
Mean	1.07 (- 5.61) a	0.39 (- 6.106) b	

Table F3: Point Quadrant results for each Clone and section of the block. Calculated standard error (SE) is in brackets. P values and LSD are also shown.

		% Gaps	Leaf Layer Number (LLN)	% Interior Leaves	% Interior Clusters	% Bunch Exposure
Clone	I10V1	10 a (3.94)	1.79 a (0.15)	19.62 a (3.93)	41.01 a (7.03)	58.99 a (7.03)
	Penfolds	19 a (5.67)	1.36 a (0.17)	11.50 a (2.87)	48.26 a (11.76)	51.74 a (11.76)
	G9V7	5 b (2.24)	1.64 a (0.13)	15.40 a (2.23)	29.17 a (5.99)	70.83 a (5.99)
P Value		*0.074*	*0.147*	*0.197*	*0.303*	*0.303*
LSD		*12.16*	*0.44*	*8.97*	*25.05*	*25.05*
Section of block	Top	10 a (10.00)	1.62 a (0.29)	14.22 a (4.62)	35.52 a (9.80)	64.48 a (9.80)
	Mid Top	10 a (5.16)	1.65 a (0.25)	17.41 a (3.34)	42.08 a (14.46)	57.92 a (14.46)
	Middle	13 a (3.10)	1.64 a (0.11)	15.69 a (3.13)	44.13 a (8.32)	55.87 a (8.32)
	Mid Bot	10 a (4.47)	1.43 a (0.19)	14.51 a (4.48)	31.55 a (9.85)	68.45 a (9.85)
P Value		*0.946*	*0.859*	*0.950*	*0.814*	*0.814*
LSD		*16.35*	*0.5739*	*11.64*	*31.62*	*31.62*
Whole Block Mean		***11.33 (2.57)***	***1.60 (0.09)***	***15.50 (1.83)***	***39.48 (5.03)***	***60.52 (5.03)***

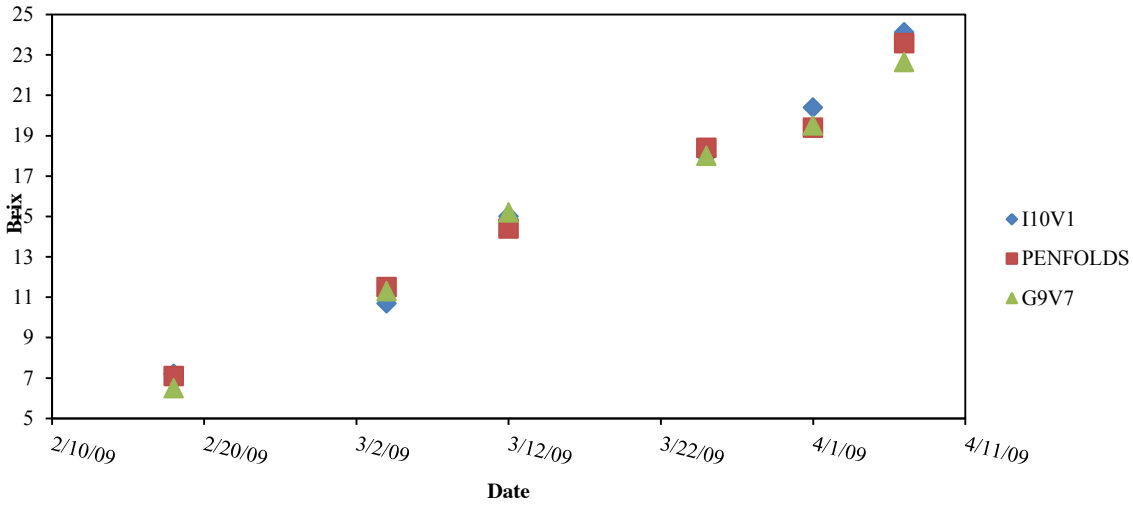

Figure F1: Mean total soluble solids (°Brix) measured during ripening up until harvest. Readings are for each of the clones I10V1, Penfolds, and G9V7.

Table F4: Summary of juice from harvested bunches showing values for total soluble solids, titratable acidity and pH. Data is grouped according to treatment and vigour category.

Treatment	Clone	Total Soluble Solids (Brix)	Titratable Acidity	pH
Flowering	I10V1	24.12	10.52	2.95
	G9V7	22.66	10.93	2.90
	Penfolds	24.66	10.45	2.92
PBC	I10V1	24.12	9.43	2.96
	G9V7	22.62	9.96	2.96
	Penfolds	22.95	10.31	2.92

Table F5: Mean total soluble soilds (°Brix) of juice from harvested bunches according to treatment and vine vigour. An unbalance ANOVA was completed to determine if there was any significant effect according to vine vigour and spary treatment (residual df = 49). The calculated P values frmm the ANOVA are as follwes:- vigour P = 0.645; fungicide treatment P = 0.363; interaction P = 0.599.

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	22.70	23.34	23.01
Low - Medium	24.70	23.00	23.91
Medium	23.12	23.52	23.32
Medium - High	23.59	22.62	23.27
High	24.25	23.47	23.87
Mean	23.69	23.19	

Table F6: Mean titratble acidity of the juice from the harvested bunches according to vine vigour and fungicide treatment. An unbalanced ANOVA was completed to determine if there were significant effects according to vine vigour and spray treatmet (residual df = 49). Th calculated *P* values and least significant dfference (lsd) (in parenthesis) are as follows:-vigour *P* = 0.063; fungicide treatment *P* = 0.005; interaction *P* = 0.427. The lsd is represented in the table via letters, where there was a signifncat difference. Letters which are not the same are significantly different.

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	10.52	9.28	9.91
Low - Medium	11.33	10.46	10.91
Medium	10.22	9.95	10.09
Medium - High	10.71	10.15	10.43
High	9.79	9.94	9.82
Mean	10.50 a	9.97 b	

Table F7: Mean pH of juice from the harvested bunches according to vine vigtour and fungicide treatment. An unbalanced ANOVA was completed to determine if there were significant effects according to vine vigour and spray treatmet (residual df = 49). Th calculated *P* values and least significant dfference (lsd) (in parenthesis) are as follows:-vigour *P* = 0.145; fungicide treatment *P* = 0.302; interaction *P* = <0.001 (0.086. The lsd is represented in the table via letters, where there was a signifncat difference. Letters which are not the same are significantly different.

	Fungicide Treatment		
Vigour	Flowering	PBC	Mean
Low	2.88	2.97	2.93
Low - Medium	2.88	2.93	2.91
Medium	2.91	2.96	2.94
Medium - High	2.91	2.93	2.92
High	3.09	2.91	3.00
Mean	2.93	2.94	

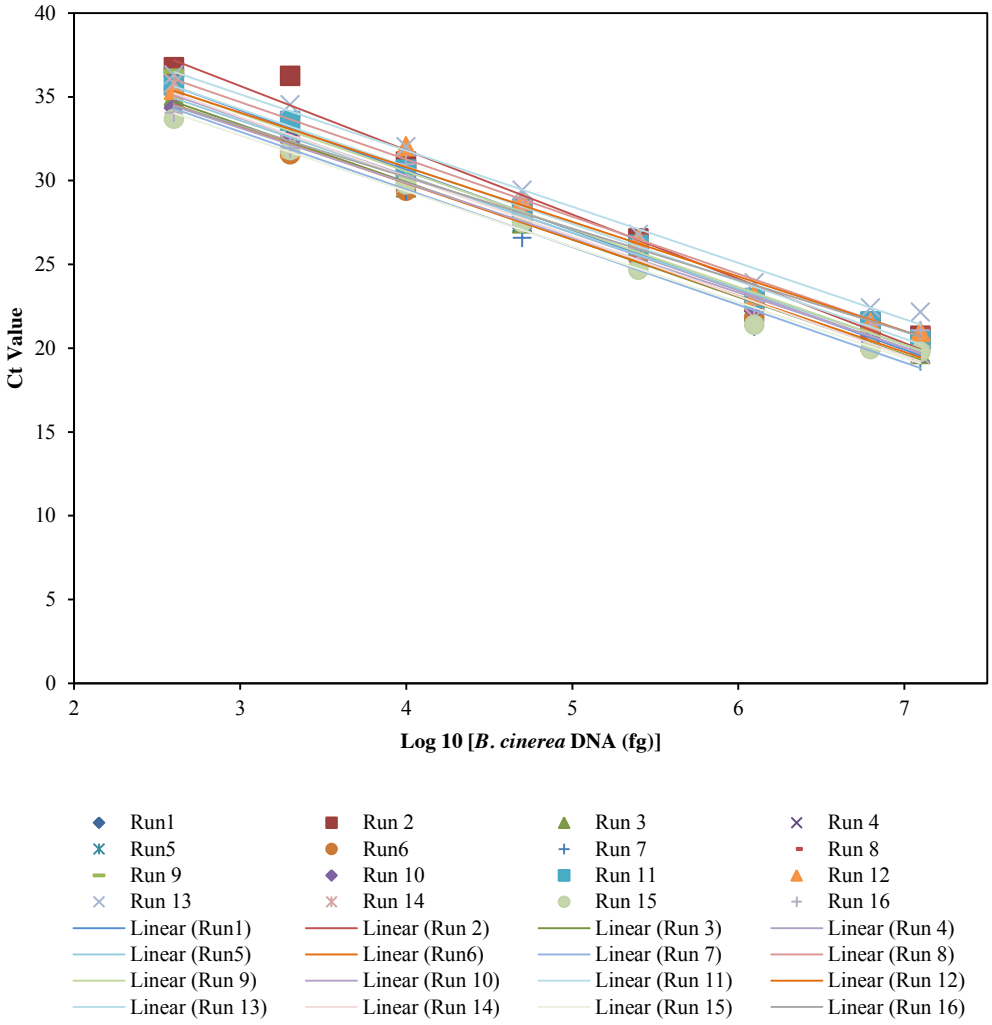

Figure F2: Regression lines for the standard dilution series used for the quantification of *B. cinerea* DNA. Ct values plotted against the log-transformed value of the DNA standard

Table F8: Linear Regression calculations of the standard dilutions series for each qPCR run. Calculated values are the R^2 value, slope, flowering intercept, the reaction efficiency and standard error of the mean (SE). All curves were significant for 8 observations.

qPCR Run	R^2 Value	Slope	y intercept	Reaction Efficiency (%)	SE
Run 1	98.5	- 3.571	44.906	91	0.723
Run 2	97.5	- 3.830	47.130	82	1.01
Run 3	99.6	- 3.432	43.646	96	0.352
Run 4	98.9	- 3.423	43.995	96	0.592
Run 5	98.3	- 3.345	43.615	99	0.708
Run 6	98.4	- 3.358	43.282	99	0.691
Run 7	99.0	- 3.442	43.237	95	0.552
Run 8	99.4	- 3.417	44.929	96	0.447
Run 9	98.0	- 3.453	44.359	95	0.802
Run 10	99.3	- 3.273	42.956	102	0.435
Run 11	99.6	- 3.423	44.539	96	0.367
Run 12	97.8	- 3.259	43.829	103	0.804
Run 13	99.1	- 3.355	45.211	99	0.520
Run 14	98.9	-3.556	44.449	91	0.626
Run 15	98.9	- 3.317	42.645	100	0.579
Run 16	98	- 3.070	42.482	112	0.709

Table F9: Converted ASIN mean percentage (%) of *B. cinerea* DNA quantified in berry samples. A unbalanced ANVOA was completed to determine if there was a correlation with Chardonnay clone and or fungicide treatment in the amount of *B. cinerea* DNA quantified (residual df = 291). The calculated *P* values and least significant difference (lsd) are as follows: - clone *P* = 0.630; spray treatment *P* = 0.348; interaction *P* = 0.004 (0.313). The lsd is represented in the table via letters, where there was a signifncat difference. Letters which are not the same are significantly different.

	Fungicide treatment		
Clone	Flowering	PBC	Mean
I10V1	0.350 bc	0.317 bc	0.334
Penfolds	0.787 a	0.248 c	0.517
G9V7	0.327 bc	0.589 ab	0.459
Mean	0.476	0.379	

Table F10: Mean monthly temperature and relative humidity readings for vineyard site for the ibuttons present in the canopy and the main weather station. Mean monthly minimum, maximum and overall mean were calculated for both temperature and relative humidity. Data for January show both before and after moving ibuttons.

	Month	Down slope – North			Upslope- Northern End			Middle			Upslope – West			Down Slope – West			Main Station		
		Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Temp (°C)	NOV	7.6	21.9	13.3	8.7	20.8	14.0	8.0	21.0	13.0	7.6	23.7	13.7	7.7	20.4	13.0	4.1	30.6	13.3
	DEC	7.8	25.9	15.5	8.5	24.9	15.5	8.7	27.0	16.0	8.4	28.6	15.9	7.8	24.7	15.3	4.9	30.0	14.1
	JAN	8.3	23.2	15.2	8.9	24.6	15.8	9.0	23.8	15.6	8.3	27.7	15.8	8.2	23.4	15.2	6.5	33.3	16.6
	JAN	10.	28.6	18.5	11.3	30.4	19.1	10.5	33.2	19.3	11.0	32.1	18.7	10.3	31.4	19.0	-	-	-
	FEB	9.9	27.8	16.8	11.0	30.0	17.5	10.4	31.1	17.5	10.1	29.2	16.6	9.7	29.9	17.2	4.7	29.1	15.6
	MAR	10.0	26.7	15.9	11.0	29.5	16.7	10.6	28.6	16.3	11.4	28.1	16.3	10.2	26.5	16.0	6.3	25.8	15.0
	APR	8.2	23.6	14.3	8.8	28.9	15.4	8.6	25.6	14.9	8.8	22.4	14.3	8.0	23.8	14.3	8.8	25.0	16.6
RH (%)	NOV	-	-	-	44.7	96.7	74.4	48.0	97.4	76.2	41.6	96.6	73.0	48.8	95.6	75.5	22.7	100.0	76.9
	DEC	38.3	91.9	66.4	34.4	94.3	64.9	32.7	92.6	64.0	31.6	91.4	63.9	39.0	96.0	66.6	26.8	100.0	69.7
	JAN	35.3	84.8	60.2	36.6	91.9	60.7	33.1	85.1	59.3	30.3	85.2	59.0	36.7	86.7	60.9	17.8	100.0	65.2
	JAN	31.3	95.4	62.7	28.8	91.7	61.2	23.2	96.3	61.4	28.6	91.4	62.2	27.4	94.3	61.5	-	-	-
	FEB	37.7	96.3	71.4	33.4	92.9	69.3	31.6	97.1	70.5	33.4	89.0	67.1	34.2	95.8	70.1	33.3	100.0	75.4
	MAR	41.7	99.1	76.2	36.6	96.8	73.6	36.9	99.1	75.4	41.5	96.2	76.4	41.2	98.9	75.4	23.9	100.0	78.0
	APR	48.3	95.2	76.1	35.2	93.3	73.0	39.5	95.7	75.3	50.9	92.0	75.7	47.4	97.1	76.1	47.4	100.0	77.7

Table F11: Summary of Temperatures for field trial. All values calculated using the data collected as of the 8th January after the moving of loggers from previous positions.

Weather Station	MGT [a]	MJT	Mean RH
Down slope- North	16.7	18.5	71.61
Upslope - North	17.4	19.0	70.78
Middle	17.3	19.3	70.76
Down slope- South	17.0	19.1	69.29
Upslope- South	17.1	18.7	70.37
Main Station	15.0 [a]/15.9	16.6	74.09

[a] Mean temperature from November- April