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Aureobasidium pullulans as potential biocontrol agent against *Eutypa lata* in grapevine trunk diseases: *in silico*, *in vitro* and *in planta* analyses

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Abstract

Eutypa lata is one of the predominant causal agents of grapevine trunk disease (GTD). This fungal pathogen can cause severe disease of grapevines and results in reduced yield or plant death. GTDs exist widely in New Zealand and their influence is getting more significant by year.

Aureobasidium pullulans is a fungal endophyte that naturally colonizing grapevines and has been used as a biological control agent in other crops. *A. pullulans* shows proven efficacy reducing severity of fungal infections against GTD pathogens such as *Diplodia seriata*. In this study, four *A. pullulans* strains were purchased and tested *in vitro* and *in planta* for their potential of inhibiting growth and movement of *E. lata* in grapevines.

In silico study the abundance of *A. pullulans* in the total fungal microbiome varied among vineyards. Contemporary-managed vineyards and Pinot Noir have higher *A. pullulans* abundance than future-managed vineyards and Sauvignon Blanc. A high abundance of *A. pullulans* reduced biomass of *E. lata*, but neither of two species has a direct association with GTD symptoms.

In vitro and *in vivo* results suggested *A. pullulans* has potential to inhibit *E. lata* but further studies were required. *A. pullulans* has significantly reduced colony size of *E. lata* when co-inoculated on Potato Dextrose Agar than *E. lata* was cultured alone. This effect existed for all four *A. pullulans* strains. When co-inoculated with *E. lata*, *A. pullulans* ICMP 3057 and ICMP 21143 was associated with increased leaf presence compared to grapevine canes inoculated with *E. lata* alone. However, no statistical evidence supported *A. pullulans* conferring a reduced stain size on cane samples.

Keywords

Aureobasidium pullulans, *Eutypa lata*, grapevine trunk disease, biological control agent, PCR, real-time PCR, antagonism test, ANOVA

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Abbreviations

GTD	Grapevine trunk disease
GTP	Grapevine trunk pathogen
GLD	Grapevine leafroll disease
VE	Vineyard Ecosystem
NGS	Next-generation sequencing
PGPR	Plant growth-promoting rhizobacteria
ELISA	Enzyme-linked immunosorbent assay
OTU	Operational taxonomic unit
GTP	Grapevine trunk pathogen
PCR	Polymerase chain reaction
dsRNA	Double-strand RNA
PDA	Potato dextrose agar
bp	Base pairs
dpi	Days post inoculation
PFR	The New Zealand Institute for Plant and Food Research Limited
CTAB	Cetyl trimethylammonium bromide
ITS	Internal transcribed spacer
MPI	Ministry for Primary Industries
ICMP	International Collection of Micro-organisms from Plants
NCBI	National Center for Biotechnology Information
HTS	High throughput sequencing

1 General Introduction

1.1 The New Zealand grape and wine industry

Grapevines (*Vitis vinifera*) are an important part of New Zealand's horticulture industry. New Zealand wine reached a record $NZ1.87 billion export for the year to June 2019, and in the past 20 years has experienced an average annual export growth of 17% (Wine 2019). There are 697 wineries in 2018 and the land cover of the vineyard has been increasing for the last nine years. Since the 1980's New Zealand has focused on producing premium wines and is now ranked as the tenth-largest wine exporter worldwide, in terms of economic value, despite contributing only 1% of the total wine produced worldwide. Wine export is the second-largest horticultural export industry, following behind kiwifruit (PFR 2017).

To combat pest and disease pressure the grapevines grown in New Zealand have changed dramatically since this exotic plant was first introduced to New Zealand in the 19th century by European settlers (This, Lacombe et al. 2006). The initial cultivars introduced were *Vitis vinifera* seedlings but these were susceptible to attack by the insect Phylloxera (see Section 1.2). Seedlings were replaced by grafted plants with hybrid rootstocks that included genetic resistance to Phylloxera from the North American cultivar, *Vitis labrusca* (King and Buchanan 1986). Today, the New Zealand wine grape industry faces new threats collectively termed grapevine trunk diseases (GTDs). Since no genetic resistance is known in the Vitis species, other treatments, including biocontrols, are required to manage them.

1.1.1 Vineyards in New Zealand

Climate to grapevines

Grapevines rely on a suitable climate to ensure high-quality production. Landowners need to consider their climatic conditions and select appropriate cultivars. Two predominant factors affecting cultivar selection are temperature and rainfall (Neethling, Barbeau et al. 2012). The wine grape growing regions across the North and South islands grow different grapevine cultivars because the climate in New Zealand varies among regions. The major

grapevine growing regions of New Zealand and their dominate grapevine cultivars are listed in Table 1.1.

Most grapes grow well where average temperatures range from 13 to 24°C during the growing seasons, and wine grapes normally require slightly cooler temperature (13-21°C) for them to accumulate sugar (Wilson 2007). Wine grapes growing in relatively warm areas results in changes in secondary metabolite production, such as polyphenols and volatiles. In addition, high temperature represses anthocyanin accumulation (Ferrandino and Lovisolo 2014). These changes negatively affect wine quality.

Table 1.1. Main grapevine growing regions in New Zealand and their predominant grape cultivars. Regions were ordered from north to south.

Vineyard region	Main cultivars
Northland	Chardonnay, Pinot Gris, Viognier
Auckland	Syrah, Chardonnay, Cabernet
Gisborne	Chardonnay, Pinot Gris
Hawke's Bay	Cabernet, Merlot, Syrah, Chardonnay, Pinot Noir
Nelson	Pinot Noir, Chardonnay, Sauvignon blanc
Marlborough	Sauvignon blanc, Pinot Noir, Chardonnay
Canterbury	Pinot Noir, Chardonnay
Central Otago	Pinot Noir, Chardonnay, Sauvignon blanc

Water is another key abiotic factor affecting grapevine and wine quality. Grapevines generally require 600 mm to 700 mm of rainfall during the growing season (Ferrandino and Lovisolo 2014). Mild and regulated water deficit has been successfully applied to balance grapevine growth and berry quality (Chaves, Santos et al. 2007). Once plants have acclimated to the moderate water deficit, they have lower cell turgor and increased berry sugar concentration (Davies, Shin et al. 2006).

New Zealand has ten fundamental wine-producing regions, ranging from Northland to Central Otago (Figure 1.1). Being surrounded by Southern Pacific, the climate of New Zealand is more moderate compared to traditional European wineries. Most vineyards are located on the eastern coast, which is relatively drier than the western coast despite the

maritime climate, which brings a high annual rainfall to New Zealand. The climate of Marlborough region (in which some research is undertaken during this MSc research) is regarded as dry in New Zealand (Figure 1.2). Unlike most areas, Marlborough has hot and dry summers but its winters can be relatively cold. The rainfall in Marlborough area varies significantly from place to place. This effect is more significant along the Wairau Valley where the nearby Mount Richmond works as a shelter reducing annual precipitation in the Wairau Valley, thus maintaining a suitably dry region for growing wine grapes (Pascoe 1983). In summer of 2017-2018 (December to February), the total rainfall in Marlborough was 283 mm. By comparison, the annual rainfall of the Auckland region ranges from 1101 mm to 1454 mm (NIWA 2018).

Low average annual rainfall has another advantage: it maximises average total sunshine hours. Marlborough is one of the sunniest places within New Zealand (Pascoe 1983), grapevines have a longer time for photosynthesis and accumulate saccharide than other regions.

Figure 1.1. Wine growing regions of New Zealand (Wine 2019). Each orange point represents a main grape growing region.

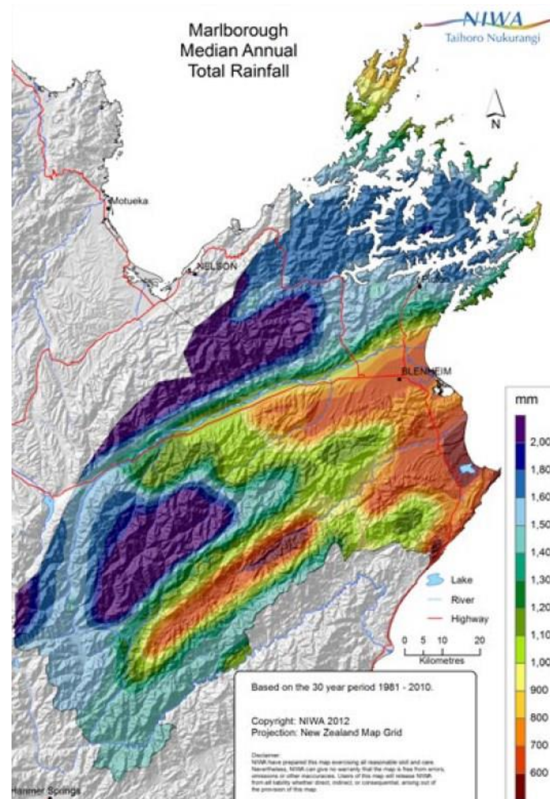


Figure 1.2. Annual total rainfall at Marlborough region. P.R.Chappell, NIWA, 2016

1.1.2 Grapevine varieties in New Zealand

There are approximately 50 grapevine cultivars commercially grown in New Zealand. Growers have developed grapevine varieties by selective breeding to fit the unique environmental conditions in different regions. Some principle varieties include Chardonnays, Pinot Gris, Merlot, Pinot Noir and Sauvignon blanc. A Marlborough Sauvignon blanc was the variety that claimed New Zealand's first award-winning wine. Soon thereafter, Sauvignon blanc became the most widely grown variety nationwide: currently, Sauvignon blanc comprises 73% of New Zealand's wine production (NewZealandWine 2018).

Most grapevines in New Zealand were planted within the last 30-40 years. In the 1980s the National government announced a controversial vine-pull scheme, encouraging growers to remove grapevines to release a glut of low-value varieties. A large proportion of grapevines in Marlborough were infected with Phylloxera, and this scheme helped to eliminate such infections. Müller-Thurgau and Riesling were replaced by Phylloxera-resistant**,** grafted Sauvignon blanc vines that produced a unique flavour in the Marlborough environment. To

maintain success, the New Zealand wine industry needs to continue to take proactive stances towards disease management.

1.2 Diseases infecting grapevines in New Zealand

New Zealand has one of the strictest biosecurity regulations in the world but phytopathogens have still managed to distribute widely through this country. The Ministry for Primary Industries (MPI) conducts inspections on all imported plant materials to ensure they do not breach New Zealand's biosecurity. Many pathogens do not cause significant symptoms early in infection, making visual identification of their presence difficult to detect (Teulon and Stufkens 2002). Despite, and perhaps prior to these efforts being established, grapevine pathogens entered New Zealand vineyards either on or in plants, fruit, insects, soil or wind.

Phytopathogens affecting grapevines in New Zealand result in economic losses. These phytopathogens include bacterial, fungi and virus which are present in most commercial vineyards and diminish vineyard longevity (Table 1.2). Infection rates of grapevine diseases vary between vineyards: from only a few individual plants to a high proportion of a block. Among these diseases that challenge grapevine health, GTDs are the most destructive infections and currently, there is no treatment against GTDs.

Table 1.2. List of some of the most damaging grapevine pathogens present in New Zealand.

Name	Pathogen classification	Causal agent of GTD?	Reference
Crown gall (*Agrobacterium vitis*)	bacteria	no	Young et al., 2003
Grey mold (*Botrytis cinerea*)	fungus	no	Beever, Laracy, & Pak, 1989
Powdery mildew(*Uncinula necator*)	fungus	no	Azzam, Gonsalves & Golino, 1991
Eutypa die-back (*Eutypa lata*)	fungus	yes	Mundy, 2010
Botryosphaeria die-back (*Botryosphaeria spp.*)	fungus	yes	Mundy, 2010

Grapevine leafroll diseases (*grapevine leafroll associated viruses*)	virus	no	Charles, et al., 2006

1.3 Grapevine trunk diseases (GTD)

Grapevine trunk diseases (GTD) refer to a series of fungal infections and normally result in severe economic loss. Their symptoms often contain weakness of grapevine growth, cane die-back and staining or browning of trunk tissues (Mundy, 2010). GTDs distribute worldwide and have caused a significant reduction of yield in vineyards in grape growing countries. The estimated annual cost of vine replacement caused by GTDs reaches 1.5 billion US dollars in all countries (Hofstetter, Buyck et al. 2012).

The first certain description of a GTD was in late 19th century, but the history of GTD is likely as long as the history of viticulture (Larignon, Fontaine et al. 2009, Bertsch, Ramírez - Suero et al. 2013). Within New Zealand vineyards GTDs are not fresh threats but their impacts are becoming more significant as our average vineyard age reaches 20 to 25 years old. GTD symptoms become more severe when plants get older, which means our aging vineyards in New Zealand are facing an increasing risk (Mugnai, Graniti et al. 1999). Symptoms are frequently detected in old vineyards and there is no known management solution to cure the disease once symptoms are identified; ultimately the vine will die in part or in total (Kaplan, Travadon et al. 2016). Management includes preventing infection and removing symptomatic or dead vines which are then replaced with new vines.

To the wine industry, the most influential effect of GTDs is grapevine death and relevant yield loss. Besides these dramatic symptoms, GTDs can cause uneven berry maturity, which results in reduced product quality. (Fontaine, Pinto et al. 2016).

1.3.1 Classifications and symptoms of GTDs

GTDs are slow perennial diseases, their symptoms normally appear on old vines and are hard to identify early on in the infection (Fontaine, Pinto et al. 2016). Since many of our vineyards were planted more than 30 years ago and the incidence of GTD symptoms are increasing across the country (Mundy 2010).

Early indications of GTD infection include leaf chlorosis and foliar wilt. This is caused by blockages of water transport within the xylem (Mundy and Manning 2010). In most cases, these vessel blockages were produced by plants to prevent further infections. Since water shortage in foliar tissues is the key factor affecting symptoms, vines often show reduced severity with sufficient irrigation even when affected by GTDs (Sosnowski, Lardner et al. 2007). Under abiotic stress (e.g. drought, freezing and nutrient deficiency), symptoms become obvious and can be very destructive (Mundy and Manning 2010).

For all types of GTDs, their causal agents are often found as a mixture of pathogens in an infected grapevine. It is rare to identify only one causal agent present per vine or vineyard (Kaplan, Travadon et al. 2016). Industry management of trunk diseased vines is to either o rework the trunk of the vine with surgery to cut out the section of fungal-infected wood or to wait and remove the whole vine when it dies. Since there is no treatment the best management method is to prevent the spread of causal agents from when the vine is young (Mondello, Songy et al. 2018). Fortunately, New Zealand is free of an Esca disease complex for which *Phaeomoniella chlamydospora*, *Phaeoacremonium* species and *Fomitoporia* spp are the reported causal organisms (Mundy 2010).

Eutypa lata is the major causal agent of Eutypa die-back (Table 1.2). It has a wide host range and, like many fungal pathogens, is very active in warm and humid climates (Sosnowski, Luque et al. 2011). Its main transmission methods are through the release of ascospores that are transported by wind and rain (Carter 1991). Initial symptoms of Eutypa die-back start from wounds (particularly pruning) with necrotic cankers on woody tissues that then move on to other tissues (Figure 1.3). It also causes stunted shoots and small, chlorotic leaves, eventually resulting in plant death approximately five years after the first symptom observed (Bertsch, Ramírez‐Suero et al. 2013).

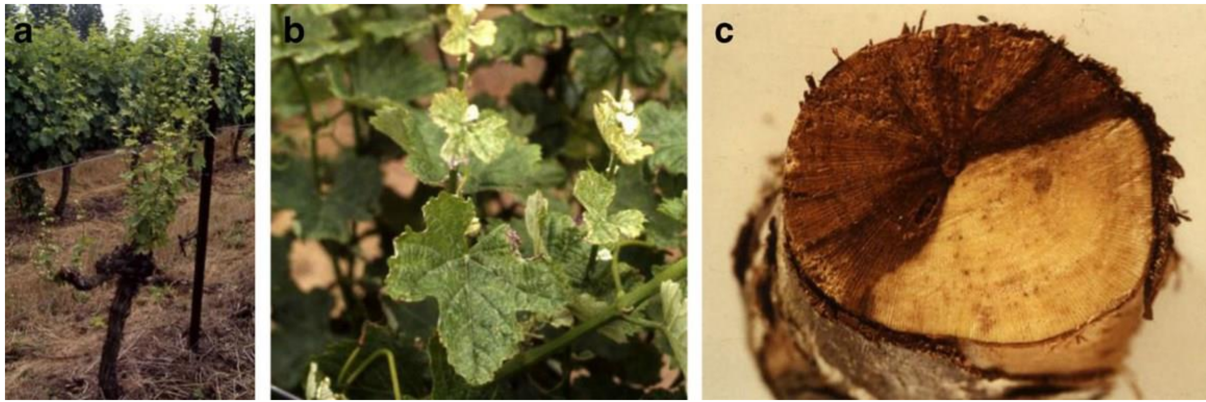


Figure 1.3. Typical symptoms of Eutypa die-back on grapevines. (a) Stunted shoots and small, unhealthy leaves symptoms of Eutypa die-back symptoms. (b) Necrosis and chlorosis on leaves. (c) Cross-section of wood showing significant discoloured tissue. Phillippe Larignon (2009).

Another common GTD Botryosphaeria die-back (Table 1.2) has a range of causal agents including more than 20 fungi, with the majority of them being opportunistic pathogens and the most common being *Botryosphaeria obtuse* (Bush 2009). The mechanisms and life cycle of Botryosphaeria die-back requires more study, but the transmission is similar to Eutypa die-back: infection commonly occurs through cuts in the vine during grafting or pruning (Úrbez-Torres and Gubler 2009). Symptoms of Botryosphaeria die-back on the trunk are barely distinguishable from Eutypa die-back and may occur in the same trunk as a mixed infection. When only Botryosphaeria dieback disease is present there may be several streaks of small black spots when the trunk is observed in cross-section (Figure 1.4). It may also contain discoloured arch-shaped lesions (Figure 1.4).

A more specific symptom of Botryosphaeria die-back is the commonly observed bud mortality. Bud mortality often indicates an infection of fungus in the young shoots. Some shoots may rupture and collapse or even die back before breaking (Niekerk, Fourie et al. 2006). Morphological identification is not sufficient to diagnose GTDs caused by Botryosphaeria because the symptoms appear very similar to Eutypa die-back and there may be a combination of fungal infections (Amponsah, Jones et al. 2011).

Figure 1.4. Symptoms of Botryosphaeria die-back on trunk. Left: small and either black or brown spots; Right: arch-shaped lesion in vascular tissue. J.M. van Niekerk et al, 2006**.**

Esca die-back (or Esca disease) is caused by a complex of fungal infections (Surico 2009). Symptoms of Esca die-back on the trunk often include discolouration and spots (Figure 1.5). Like other trunk diseases, Esca die-back usually causes foliar symptoms, such as a tiger-like colour pattern on leaves. Fortunately, Esca die-back has not been reported in New Zealand.

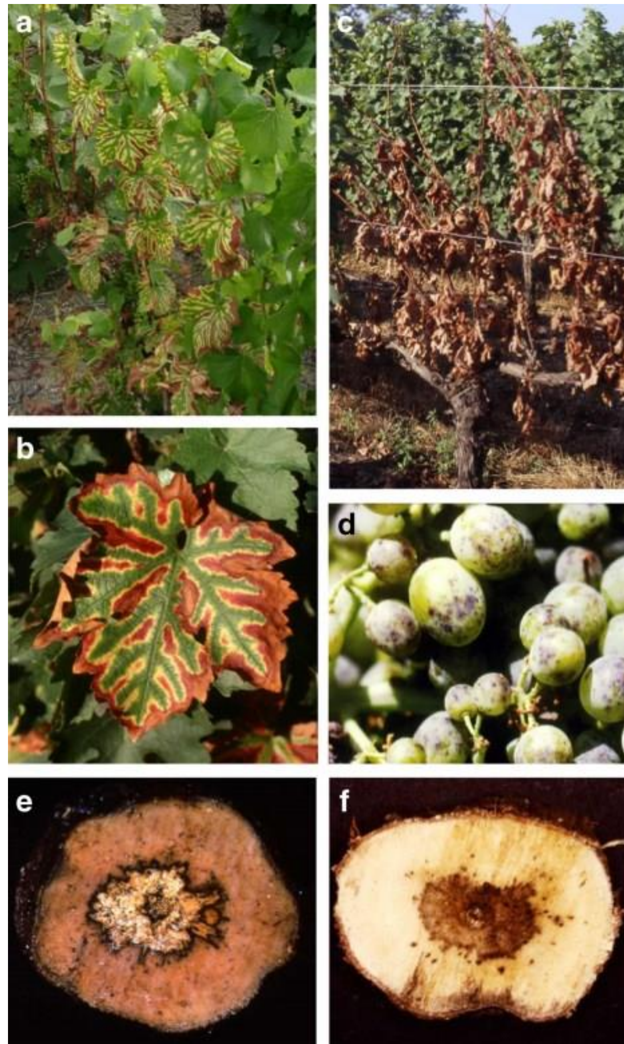


Figure 1.5. Typical symptoms of Esca die-back. (a, b) tiger-like necrosis and discolouration on leaves. (c) Dead shoots of infected vines. (d) Spotted berries. (e,f) white rot and black wood streaking on the cross-section. J.M. van Niekerk et al, 2006.

1.3.2 Physiological responses of grapevine to GTDs

Grapevines respond to grapevine trunk pathogen (GTP) infection by multiple pathways. These pathways include secreting lignin, producing teloses and induce plant defense system.

An initial protection method of grapevines is by increasing lignin components in cell walls (Rolshausen, Greve et al. 2008). Most initial infections of GTPs occur at a wound site on a young plant during grafting or pruning. Therefore, the plant response to wounding plays an important role in the pathogen's and plant's development. In the wound area, cell necrosis results in signals to nearby healthy cells that triggers a signalling response pathway (Bloch

1952). These signals may induce the accumulation of phytoalexins and other antimicrobial compounds (Belhadj, Saigne et al. 2006).

Grapevines can also respond to wounding and fungal infections by producing teloses. Formation of vascular teloses blocks the xylem near the wound or fungal infection and minimises pathogen movement within the plant (Mundy and Manning 2011). A restricted throughput of vascular tissues decreases water transport which results in water deficiency. This phenomenon is particularly obvious in young leaves and shoots. When plants are under environmental stress, foliar symptoms occur as a sign of early GTD infection, as observed for Eutypa die-back.

The plant defense system is a fundamental dimension of plant “immune response”, is a result of co-evolution between plant and phytopathogens (Toruño, Stergiopoulos et al. 2016). Broadly, plant defense system consists of microbial-associated molecular-patterns-triggered immunity (MTI) and effector-triggered immunity (ETI) (Muthamilarasan and Prasad 2013). Plants have a capacity to recognize and respond to specific pathogens by activating host resistance genes as a response to fungal infection (Dodds and Rathjen 2010). Detailed steps of plant defense system contain much molecular science and not included in this section.

1.3.3 *Eutypa lata* as fundamental GTD causal agent

As detailed earlier, *E. lata* is an important GTP which causes Eutypa dieback and often exist with other GTPs. *E.lata* is often transmitted as by ascospores by pruning (Munkvold and Marois 1995). *E. lata* infects vascular vessels and moves slowly into other parts of the trunk damaging nearby tissues by secreting a phytotoxin (Carter 1991, Rolshausen, Greve et al. 2008). Its pathogenicity results from chemical products such as enzymes, phytotoxin and some other secondary metabolites. Gradually grapevines express multiple symptoms within the same plant and eventually die after two or three years.

Hydrolytic enzymes expressed by *E. lata* are basic components that degrade grapevine trunk cell walls (Octave, Amborabé et al. 2006). Apart from enzymes produced by other non-pathogenic fungi, the starch degrading enzymes, glycosidase and phenol oxidase are the

most studied enzymes related to pathogenicity (Rolshausen, Greve et al. 2008). The consequence of these enzymes is that *E. lata* significantly decreases hemicellulosic xylose and glucose within plant cell walls when inoculated into grapevines (Merlot and Cabernet Sauvignon) and degrades starch stored in trunks (Rolshausen, Greve et al. 2008). These enzymes provide nutrients that support the growth of the pathogen but result in weakened plants both structurally and energetically.

The phytotoxin that *E. lata* produces kills plant cells. Eutypine is the main toxin produced by *E. lata*. It targets the plant cell plasma membrane and plays an important role in disease development (Amborabé, Fleurat-Lessard et al. 2001). Known as a weak lipophilic acid, eutypine accumulates in the cytoplasm of plant cells and affects membrane functions (Deswarte, Eychenne et al. 1996). In an i*n vivo* study eutypine was shown to induce a significant drop in cell mitochondrial respiration (Kim, Mahoney et al. 2004). *E. lata* strain or environmental diversity contributes to which secondary metabolites are produced by *E. lata. E. lata* strains isolated from Europe normally produce eutypine while those isolated from Australia and New Zealand are more often found synthesizing eutypinol or eulatinol (Mahoney, Lardner et al. 2003). In addition to affecting the plant's cytomembrane functions, the growth and respiration of yeasts (e.g. *Saccharomyces cerevisiae*) are also inhibited by the secondary metabolites synthesised by *E. lata* (Kim, Mahoney et al. 2004).

E. lata provides the most simple model phytopathogen of GTD present within New Zealand and therefore was chosen as a phytopathogen for research within this thesis (see section 1.6). Its prevalence was determined in commercial vineyards (Chapter 2), it was used to test a potential biocontrol activity within *in vitro* antagonism tests (Chapter 3) and *in planta* protection tests (Chapter 4).

1.3.4 Diseases management of *E. lata* related GTDs

There is no effective treatment to grapevines with GTDs detected. Current approaches include either to prevent GTPs infection and development, or remove unhealthy vines to reduce loss and protect other plants from infection (Mundy and Manning 2010).

Wound protection of grapevine is of vital importance in preventing the spread of fungal diseases, such as trunk diseases. Researchers soaked rootstock and scion cuttings in the water and sterilizing agents such as halogenated alcohols, benomyl, and captan, the total pathogen incidence dropped from 30% to 13.5% (Fourie and Halleen 2006). A study aiming at taking place of artificial chemicals by hot water (50°C) resulted in failure (Rooney and Gubler 2001), despite hot water treatment in nurseries works well dealing with many pests.

1.4 The Vineyard Ecosystems programme

This thesis research is aligned with the Vineyard Ecosystems (VE) Programme. The VE Programme is funded by New Zealand Winegrowers and the Ministry of Business, Innovation and Employment (MBIE). The programme aims to determine how vineyard management impacts on the biodiversity of commercial vineyards within New Zealand. This study is carried out on 12 white and 12 red grapevine blocks in commercial vineyards at Marlborough and Hawke's Bay. The entire programme is divided into three research aims: "The vineyard as an ecosystem", "Relating under-vine management, biota and leafroll virus" and "Pathogen management". These three research aims form a comprehensive view of the grapevine ecosystem including its microbiota and how they interact within grapevines with the aims to improve vineyard management, provide eco-credentials and increase vineyard longevity. Important to this thesis project, the VE project collected data in commercial vineyards in two major grapevine growing regions of South Island and North Island, Marlborough (Figure 1.6) and Hawke's Bay. The vineyards grew either Sauvignon blanc or Pinot noir in Marlborough and Sauvignon blanc or Merlot in Hawkes Bay.

Figure 1.6. Approximately indication of vineyards location in Marlborough area (red zone).

1.4.1 Contemporary management and Future management

The VE programme vineyards were separated into two management systems: either "contemporary" or "future" with half of the vineyards in each region under each management system.

Contemporary treatment is representative of the way most grapes are most commonly grown in New Zealand now (2019). Growers are specifically asked to continue their vineyard management, including small annual variations in practice as considered necessary. The only consistent difference with the 'Future' treatments is that these Contemporary vineyards use herbicides to maintain a bare soil under-vine strip, and they may use synthetic fungicides or pesticides to combat fungal diseases and insect pests, and synthetic fertilisers to mitigate nutritional deficiencies. Most of these vineyards maintain an inter-row permanent ground cover. Current control approaches to fungal pathogens are fundamentally based on the palliative application of synthetic fungicides and oxidative chemicals. The most commonly isolated pathogen in trunk disease infected grapevines, *Phaeomoniella chlamydospore*, could infect rootstock as early as propagation stages (Raski, Goheen et al. 1983). Because trunk diseases often take a long period to develop and are difficult to be diagnosed at an early stage, growers may waste many years and get only limited products or even eventually dead plants. To protect the economic crop and reduce the negative effect on the environment, only chemical and mechanical treatments are insufficient.

The future treatment represents vineyard management that does not use herbicides to control weeds in the under-vine region. They use alternative means of controlling under-vine weeds, which are mainly mechanical by cultivation or mowing. They generally try to maintain a semi-permanent ground cover (inter-row) comprising a wide range of plant species. To help combat fungal diseases and insect pests, preferably naturally occurring products are used (if and when required) in these vineyards although some reserve the right to use synthetic chemistry for particular issues; there are no strict rules around the use of fungicides and fertilisers.

Management strategies have impacts on the microbiome in plants and affect the varieties and biomass of endophytes and mycorrhizal colonization of grapevine roots (Almeida, Daane et al. 2013, Gdanetz and Trail 2017). Mechanical cultivation, use of synthetic pesticides and fungicides are used to reduce the negative pest and disease factors in vineyards.

1.4.2 Vineyard ecosystems Research Aim 1.3, Pathogen management

Of particular importance to this thesis project, the VE programme Research Aim 1.3 focussed "Inside the grapevine". This Research Aim 1.3 has the major outcome for the identification of management practices that can increase vine health, and thereby vine longevity, even in the presence of detectable pathogens. Research Aim 1.3 explores the microorganisms through high throughput sequencing of a large number of grapevines per vineyard each year (to date, over three years) to identify the microbial population and determine which of them are beneficial to the health of grapevines and which are pathogenic. By analysing these microbes over time and by assessing the plant health status the aim is to identify "What factors correlate with grapevine health in the presence of pathogens?"

1.4.3 Microbial communities interactions in plant and the mechanisms of pathogen-pathogen interaction

Koch's postulates have been a classical guideline to demonstrate pathogenicity of an individual microbe and to evaluate its causal infectious disease. However, typical Koch's postulates sometimes are not sufficient to identify plant pathogens and can ignore critical interactions that cause diseases (or keep the host healthy) (Byrd and Segre 2016). One of the key limitations of these postulates is that they assume there is one pathogen as a causal agent of a specific disease. This hypothesis is in fact a double-edged sword: it is effective whereby the microbe can be isolated into a pure culture e.g. most and fungal pathogens, however, this is not always possible for some microbes, e.g. fastidious bacteria and viruses (Prescott, Feldmann et al. 2017). In addition, the postulates do not address the importance of microbial communities and their interactions, which play vital roles in pathogenicity (or plant health). Microbial communities have shown great effects on human diseases that are known as syndromes such as gut microbial-related obesity (Haro, Garcia-Carpintero et al. 2016). In these syndromes more than one microbe is required for a disease to develop (in a similar fashion to Botryosphaeria die-back). Likewise, microbial communities may have protective capacity as is being revealed by the 'gut microbiome' (Haro, Garcia-Carpintero et al. 2016). The concept of "microbial protectors" has been formed to extend our understanding of Koch's postulates (Figure 1.7). Thus, diseases may be caused by one or more microbe and likewise,

the disease may be protected against by one or more microbes, a biological control agent (BCA, see section 1.4.5) or community of agents (BSCs).

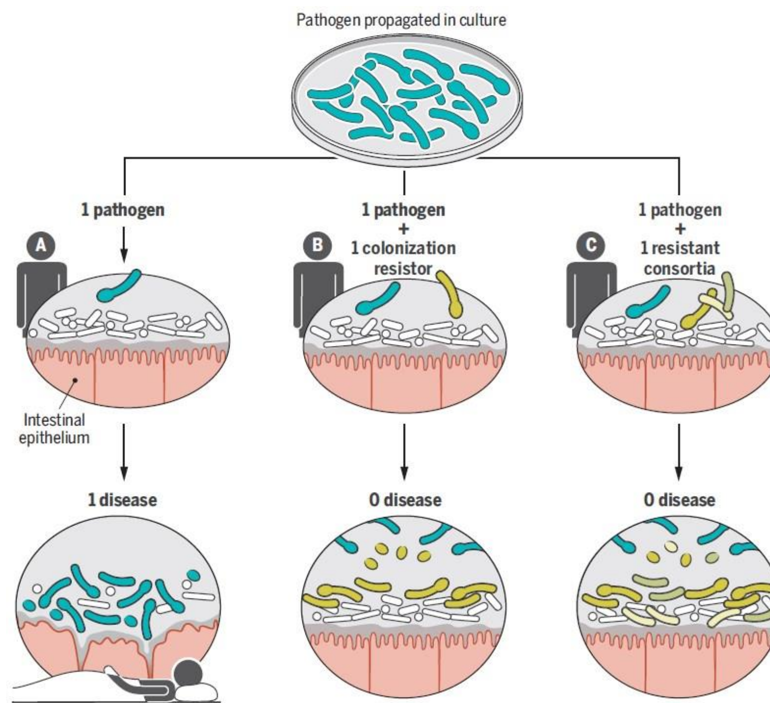


Figure 1.7. A supplement to classical Koch's Postulates in human diseases: Microbial protectors. According to initial Koch's postulates, specific pathogens in a host will include disease (A). This assumption is challenged when some organisms are present that can protect against pathogens (B). These beneficial organisms are called "Microbial protectors" in this supplemental concept. Sometimes an existence of microbial communities will result in even stronger protective effects (C) (Byrd and Segre 2016).

Within human pathology, there is an increasing awareness that pathogens often do not operate alone. However, to date, most plant diseases are more frequently studied at a monospecies level (Lamichhane and Venturi 2015). But the reality is that in many cases, fungal pathogens act together and are affecting a plant at the same time (Fitt, Huang et al. 2006). A single pathogen infection does not necessarily result in severe symptoms (Lamichhane and Venturi 2015). For example, Esca dieback and Petri disease are caused by complexes of three (or more) fungi (Table 1.3).

Synergistic pathogen-pathogen interactions enhance the pathogenicity of each individual pathogen to form a disease "complex". There are several examples in grapevines whereby

the interaction of several pathogens is synergistically leading to plant disease or increased disease severity (Table 1.3).

Table 1.3. Fungi-fungi synergistic interactions that lead to plant disease and increased disease severity in grapevines. Extracted from Lamichhane and Venturi (Lamichhane and Venturi 2015).

Disease	Causal agents	Reference
Grapevine decline	*Botryosphaeriaceae* sp. and *Ilyonectria* sp.	(Whitelaw‐Weckert, Rahman et al. 2013)
Black dead arm	*Botryosphaeria dothidea*, *Diplodiaseriata* and *Lasiodiplodia theobromae*	(Dubos, Cere et al. 2001)
Black foot	*Cylindrocarpon liriodenderi* and *C. macrodidymum*	(Halleen, Fourie et al. 2006)
Esca dieback and Petri disease	*P. chlamydospora* and *Phaeoacremonium aleophilum*	(Li, Bonneu et al. 2016)

Although many beneficial endophytes promote plant health, some endophytes establish an environment that either favours pathogen growth or increases disease severity. *Phoma* spp.are a commonly identified endophytic fungus present in a variety of plants including grapevines (Rego, Oliveira et al. 2000). Some strains from *Phoma* sp. were uncovered to support pathogens growth in Japanese knotweed (*Fallopia japonica*) and increase symptom severity of another fungus (*Puccinia polygoni-amphibii* var. *tovariae*) that causes a rust disease (Kurose, Furuya et al. 2012).

1.4.4 Rhizosphere and endophyte microbial communities and plant health

A healthy root system works as a fundamental of yield and commercial profit in vineyards. Sometimes, roots are the initial plant organ infected by pathogens or infested by pests (Salomon, Bottini et al. 2014). By contrast, the rhizosphere environment and rhizobacteria combine to form an important complex that promotes plant health (Compant, Reiter et al. 2005). The root surface microbiome often has an impact on plant adaptation to the ambient environment and promotes plant health by either decreasing diseases severity or increasing host tolerance to stress (Welbaum, Sturz et al. 2004). Thus, concepts such as soil agro-

ecosystems or suppressive soils have been developed to describe these complexities (Welbaum, Sturz et al. 2004, Zarraonaindia, Owens et al. 2015).

Endophytes are a diverse group of microbial organisms that exist within their plant host and affect (both positive and negative) plant defense and carbon cycling (Mandyam, Fox et al. 2012). Some bacterial endophytes decrease toxin synthesis and/or the severity of symptoms of pathogens and benefit their plant hosts in diverse conditions(Mousa, Shearer et al. 2016). For example, bacterial endophytes in millet roots can create a physicochemical barrier to protect hosts against the fungal pathogen *Fusarium graminearum* (Mousa, Shearer et al. 2016). Generally, the community structure of endophytic bacteria becomes more complex with the growth of plants (Gdanetz and Trail 2017).

Several endophytes, named plant growth-promoting rhizobacteria (PGPR), have positive effects on plant health (Sabir, Yazici et al. 2012). Some PGPR act by directly secreting beneficial compounds such as cytokines, auxins and even antibacterial peptides (Maurhofer, Keel et al. 1992). Sometimes their positive effects can be combined, for example, *Bacillus megatorium* and *Pseudomonas spp.* Together can produce auxins and increase phosphate solubilisation (Sabir, Yazici et al. 2012).

1.4.5 Biological control agents (BCAs) against grapevine pathogens

The cost of fungicides for disease prevention and consumers' pressure to have more environmentally friendly growing conditions demands new methods of controlling diseases. Such control methods include the use of biological control agents (BCAs).

Plant growth-promoting rhizobacteria and antagonistic fungal factors are potential methods in future growing systems. The mechanisms of BCA action include competition with pathogens for root or trunk niches and nutrients, actively increasing host nutrients intake from the soil, positive synthesis of a suppressor against phytopathogens, or indirectly by inducing the plant's defense system (see Table 1.4). For instance, in other plant pathosystems, some BCAs express detoxifiers of albicidin toxin synthesized by *Xanthomonas albilineans* (Basnayake and Birch 1995). A large number of BCAs induce systemic acquired resistance which is often broad-spectrum and long-lasting (Walters, Ratsep et al. 2013).

Isolated endophytic and rhizosphere actinobacteria from young grapevines showed anti-fungal effects by 13.8% and 16.0% respectively (Morales‐Cruz, Allenbeck et al. 2018).

Table 1.4. A list of biocontrol pathways to promote plant health (Lugtenberg and Kamilova 2009).

	Biocontrol pathway to promote plant health
1	Secrete antibiosis directly to suppress or kill pathogens
2	Compete with pathogens for niches and essential nutrients
3	Interfere with pathogen signalling pathway or metabolism
4	Induce plant resistance (defense responses)
5	Degradation of virulence factors or phytotoxins

There is increasing interest in using bio-suppression of pathogens in grapevine to reduce fungal diseases. One of the most targeted fungal pathogen: *Botrytis cinerea*, can infect numerous important crops in New Zealand including kiwifruits, apples and grapevines. For this reason, there are many studies about biocontrol methods against *B. cinerea* (Plesken, Weber et al. 2015). As a well-studied fungal pathogen, many biological control agents (BCAs) have been identified to suppress *B. cinerea*. The BCAs could be fungi, bacteria or even virus (Elmer and Reglinski 2006). However, sometimes a phytopathogen is not controlled by a single BCA. For instance, the wide-spread causal agent of grapevine grey mould, *B. cinerea* is not effectively controlled by single BCA application (Jacometti, Wratten et al. 2010). However, inoculation of a combination of three BCAs together (*Trichoderma atroviride*, *Bacillus subtilis* and *Aureobasidium pullulans*) provided effective Botrytis management on grapevines (Pertot, Giovannini et al. 2017).

1.4.6 *Aureobasidium pullulans* as a biological control agent

Auerobasidium pullulans has potential to control fungal plant diseases. *A. pullulans* is a widespread endophytic fungus that can be found in the phyllosphere and rhizosphere of a variety of crops including grapevines (Elmer and Reglinski 2006). *A. pullulans* can inhibit the growth of *B. cinerea in vitro* and *in vivo* (Schena, Ippolito et al. 1999). In addition, *A. pullulans* has shown inhibition on diseases caused by *B. cinerea* in both grapevines and

apples. *A. pullulans* can also restrict *Neofusicoccum parvum* infection in apple canker (Rusin, Di Francesco et al. 2019).

There are multiple mechanisms behind *A. pullulans*' protective effect. Like other beneficial endophytes, one possible reason is *A. pullulans* compete for space and nutrients to be predominant (Janisiewicz, Tworkoski et al. 2000). Other ideas include *A. pullulans* can secrete antimicrobial metabolites, suppress pathogens' enzyme function (TAKESAKO, IKAI et al. 1991) and induce the plant defense system (Rühmann, Pfeiffer et al. 2013). Even an autoclaved culture filtrate of *A. pullulans* significantly increases gene expression encoding resveratrol in grapevines (Rühmann, Pfeiffer et al. 2013). *A. pullulans* can also induce the biochemical defense response in apple tissues, for instance, *A. pullulans* could increase the content of chitinase and peroxidase at apple wounds. This effect plus its occupation of nutrients and space might be the major reasons of *A. pullulans* beneficial impacts (Ippolito, El Ghaouth et al. 2000). *A. pullulans* is also isolated from table grapes and has proved to have beneficial effects against grapevine bitter rot disease (Schena, Ippolito et al. 1999). As a result*, A. pullulans* strains are commercially available and are applied by fruit growers to prevent some fungal phytopathogens.

A. pullulans may inhibit pathogen growth through multiple mechanisms. The pathways by which *A. pullulans* suppresses *B. cinerea* is by either limiting the necessary requirements of fungal growth or even producing some toxic substance against pathogens (see Table 1.4). Biofilm produced by *A. pullulans* also helps to restrict pathogen development and block pathogen approach to nutrients (Klein and Kupper 2018). In addition, triggering plant defense response is another fundamental mechanism: crops (grapevine, avocado, etc.). Applying *A. pullulans* to trigger plant defense is a promising research direction further understanding is required. A possibility exists that *A. pullulans* may be a BCA for fungi other than *B. cinerea*. *A. pullulans* has also been isolated from grapevine trunks. Therefore, it is worthwhile studying the potential BCA activity of *A. pullulans* on GTDs and causal agents.

1.5 Project aims

This research aims to identify whether a microbe may keep grapevines healthy in the existence of one or more pathogens with a special interest in *A. pullulans* and *E. lata*. The

aims of this project are listed below with each forming a chapter. Each aim was carried out concurrently.

Project aim 1. Evaluate association among *A. pullulans*, *E. lata*, GTD symptoms and other variables (grapevine variety, management methods, year and location) in vineyards of VE projects.

Hypotheses:

1. The presence of *A. pullulans* in grapevines reduces the abundance of *E. lata* and decreases GTD symptoms;

2. Vineyard variables (grapevine variety, management methods, year and location) impact on the abundance or severity of *A. pullulans*, *E. lata* and/or GTD symptoms.

Null hypothesis:

1. The presence of *A. pullulans* does not affect the abundance of *E. lata* or decrease GTD symptoms;

2. Vineyard variables (grapevine variety, management methods, year and location) have no impact on abundance or severity of *A. pullulans*, *E. lata* and/or GTD symptoms.

Method: Assess two years of grapevine health status and microbiome data to correlate the associations among year, variety, management method, fungal OTUs and trunk disease symptoms.

Project aim 2: Identify the strain of *A. pullulans* that provides the best biocontrol of *E. lata* within *in vitro* conditions.

Hypothesis: One or more strains of *A. pullulans* shows the presence of antagonistic effects on *E. lata* when grown *in vitro*.

Null Hypothesis: No isolate of *A. pullulans* shows the presence of an antagonistic effect on *E. lata* when grown *in vitro.*

Method: Compare growth rates and colony radii of single and dual-cultures of *A. pullulans* (four strains) and/or *E. lata* (one strain) on solid medium.

Project aim 3: Identify the strain of *A. pullulans* that provides the best biocontrol of *E. lata* and GTD symptoms within *in planta* conditions.

Hypothesis: One or more strains of *A. pullulans* can increase 'cane health' (i.e. the presence of leaves and roots, reduction of GTD symptoms, and/or lower abundance of *E. lata*) when inoculated alone or co-inoculated with *E. lata* into grapevine canes.

Null Hypothesis: No strain of *A. pullulans* can significantly increase 'cane health' when inoculated alone or co-inoculated with *E. lata* into grapevine canes.

Method: Compare leaf and root presence, cane stain size, and fungal presence (detected by real-time PCR) following single or dual-inoculations of *A. pullulans* (each of four strains) and/or *E. lata* (one strain) into grapevine canes.

2 Relevant Vineyard Ecosystem data and identify beneficial micro-organisms

2.1 Introduction

This chapter addresses project aim 1, to "Evaluate the association between the amount of *A. pullulans* and *E. lata*, the presence or absence of GTD symptoms, and other vineyard variables (grapevine variety, management methods, year and location) within the VE programme". The research is divided into addressing two hypotheses:

1. The presence of *A. pullulans* in grapevines reduces the abundance of *E. lata* and decreases GTD symptoms;

2. Vineyard variables (grapevine variety, management methods, year and location) impact on the abundance or severity of *A. pullulans*, *E. lata* and/or GTD symptoms.

The data for analysis was provided from the VE programme (section 1.4) including the GTD symptom data from research aim 1.1 and the high throughput sequence data that identified microbes within the trunks of vines from research aim 1.3.

Over 3 years, GTD symptoms were assessed by an individual vine level, which was recorded on a vineyard map. Within each vineyard, nine loci were identified. Each locus comprised five vines and each of these were subjected to a biopsy each year (to date, for three years) and high throughput sequencing to assess the microbial population and to identify pathogens and potential BCAs, each year. Although the sampling occurred at a vine level, the high throughput sequencing was performed on a "five-vine-composite", i.e. DNA isolated from five individual vines within a row were combined for sequencing. The GTD symptoms assessment, biopsying and sequencing was performed by Dion Mundy, Bhanupratap Vanga and/or Bex Wolley. The data was analysed by Dr Beatrix Jones in response to the research questions posed through the research within this thesis.

Five key approaches drove data processing or analysis:

1. Achieve a general understanding of fungal OTU data and trunk disease symptom record. This established a model of proportion/level of key OTUs in plant microbiome (especially for *A. pullulans* and *E. lata*) for further steps. Also, classification and quantification of GTD symptoms were performed.

2. Relate GTD symptoms with *A. pullulans*, *E. lata* and other key OTUs to build the connection of OTU model we established with GTD symptoms and demonstrate how fungal OTUs affect GTD symptoms.

3. Study the interactions between *A. pullulans* and *E. lata*, with focus on the potential antagonism of one fungus against another in OTU models. This uncovered whether *A. pullulans* showed inhibition of *E. lata*.

4. Study major whether the variables of commercial vineyards (management, grapevine variety, growing year, locus location) on GTD symptoms or presence/level of *A. pullulans* and *E. lata*.

5. Determine which five-vine composites (loci) would inform the hypotheses further.

2.2 Materials and methods

2.2.1 Contributions by others in this research chapter

GTD symptoms recording was performed by Dion Mundy, Bhanupratap Vanga and Bex Woolley of PFR Blenheim. *In silico,* OTU model establishment, data analysis and summary of results including figures were performed by Beatrix Jones at The University of Auckland. I undertook an initial manual observation of fungal OTU assessment and correlation with GTD, a small number of GTD symptom assessments in the 2018/19 season, and I posed the questions of the data, along with my supervisor Robin MacDiarmid (PFR, Auckland and The University of Auckland). Detailed explanations of analysis procedures and research methods are stated in Appendix 3.

Only those methods and materials in which I was involved are presented in this section. Those used by others in the preparation of data that I used are in Appendix 2.

2.2.2 Sequencing data and grapevine health data analyses

Sequencing data was assessed as operational taxonomic units (OTUs) or to species level as required. Because our grapevine trunk NGS results contained 1753 OTUs, which was too high for most statistical models to be analysed. The 20 most commonly identified fungal OTUs (include *A. pullulans* and *E. lata*) were chosen for statistical analysis.

Three major mathematics analysis methods were used to understand the potential impact of the top twenty most commonly identified OTUs: if they are protective, pathogenic or neutral to grapevine health. All of the following analyses were based on the OTU rates (reads/total for that five-cane-composite) rather than absolute count from existing High throughput sequencing (HTS) data.

The overall test of differences between symptomatic and asymptomatic five-vine-composites was carried out by permanova (Anderson 2001). This aimed to demonstrate the frequencies of symptoms and severity of GTDs among selected vineyards.

To study individual OTUs and their effect on grapevine health status, we chose Mann-Whitney (nonparametric) tests, with control of the false discovery rate at 5% (Benjamini and

Hochberg 1995). This is a normal approach to understand how a specific variable influence the entire results. In addition, to determine the interactions between microbes, sparse PLS-discriminant analysis was applied to construct a model with a small number of OTUs. In this model, every OTU was paired with the all of the remaining OTUs and we used the implementation in "mixOmics" as described by Le Cao K (Lê Cao, Boitard et al. 2011).

2.2.3 Effects of *E. lata* and *A. pullulans* on grapevine health

For each locus, we looked up the location (vine/row/bay) and recorded GTD symptoms. Then we examined the total number of reads for the samples, all loci were separated to three groups: asymptomatic, one symptomatic vine and two symptomatic vines (no locus had more than two symptomatic vines). Then we characterized microbiota similarity of loci based on Bray-Curtis, this reflects vine health status and OTU components in general. It indicates the relationship of potential protective microbes at the same time.

The above methods provided general information on grapevine health and similarity, but they are insufficient for us to understand the variability of cultivar diversity and management systems. We then looked at a particular interaction of interest, between *E. lata* and *A. pullulans*. *E. lata* is present in 66 of 94 samples and *A. pullulans* is present in all samples. We fit a generalized linear mixed model with symptoms (present or absent) as the response, a random effect for vineyard and the OTU proportions for *E. lata*, *A. pullulans* and their interaction as the fixed effects. Permanova was applied as analyses methods.

2.2.4 Associated factors affecting the proportion of *A. pullulans* and *E. lata* and GTD symptoms

OTU counts were aggregated to species names and samples with less than 1000 total reads were excluded. Counts were divided by the total reads in each sample to create proportions.

Varieties of studied grapevines. Data were assessed to demonstrate their effect on *A. pullulans* and *E. lata* proportions. Varieties of grapevine cultivars, management systems and vineyard location were assessed in tests.

2.2.4.1 Questions studied to identify factors that affect GTD symptoms, the incidence of *A. pullulans* and *E. lata*

Rather than provide the statistical analysis, the research questions posed of the data are listed as follows.

Question 1: Does variety and/or management and/or year affect *A. pullulans* biomass, treating vineyard location as a random effect?

Question 2: Is *E. lata* affected by management or variety, while accounting for vineyard location effects?

Question 3: Is *E. lata* incidence associated with *A. pullulans* incidence, after controlling for vineyard location, management and variety?

Question 4: Is the presence of symptoms influenced by management or variety, after controlling for vineyard location?

Questions 5: Do the incidences of *E. lata*, *A. pullulans* and their interaction predict the presence of symptoms, controlling for vineyard location?

2.3 Results

2.3.1. Vineyard with special OTU symptoms development over 2 years

In the 2017/2018 season in Marlborough, the incidence of GTD symptoms ranged from 2.4% to 18.9% (Figure A3.1). Five out of twelve vineyards reported their GTD incidence in 2017/2018 season, the variations vary from site to site. By contrast, the GTD symptoms reduced significantly in vineyard 15-PNC over the years, i.e. it dropped from over 15% in season 2015-2016 to around 3% in season 2017-2018. This rapid reduction may indicate a higher likelihood of biological effect than other vineyards since the management system kept unchanged in this site. To identify such management effects a bioinformatics analysis was undertaken.

2.3.2 Frequently identified fungal species in vineyards by High throughput sequencing (HTS)

Among the large number (>1000) of detected fungal OTUs, 30 of the most frequently identified fungal species are listed in Table 2.1. It contains both pathogenic and beneficial fungi, with many of them having unclear or academically controversial effects on plants. Of note, both *A. pullulans* and *E. lata* are listed as detected fungi.

Table 2.1 Top thirty most frequently identified fungal by high throughput sequencing (HTS). OTUs were classified by their effects on the plant. Pathogenic represents fungus could normally cause disease or have negative on plant growth; beneficial represents fungus can promote plant growth or inhibit pathogen infection; controversial represents effects of fungus were recorded differently in articles or depend on plant species.

Phylum	Species	Potential effects on plant	References
Ascomycota	*Phaeomoniella chlamydospora*	pathogenic	(Álvarez-Pérez, González-García et al. 2017)
Ascomycota	*Eutypa lata*	pathogenic	(Ferreira, Matthee et al. 1991)
Ascomycota	*Phoma herbarum*	unknown	(Rivero-Cruz, García-Aguirre et al. 2000, Hamayun, Khan et al. 2009)

Ascomycota	*Cladosporium tenuissimum*	pathogenic	(Fisher and Petrini 1992)
Ascomycota	*Alternaria alternata*	pathogenic	(Hamayun, Khan et al. 2009)
Ascomycota	*Aureobasidium pullulans*	beneficial	(Klein and Kupper 2018, Rotolo, De Miccolis Angelini et al. 2018)
Ascomycota	*Fusarium cerealis*	pathogenic	(Amarasinghe, Tittlemier et al. 2015)
Ascomycota	*Sphaeropsis sapinea*	pathogenic	(Georgieva and Hlebarska 2017)
Ascomycota	*Cladosporium oxysporum*	controversial	(Raj, Manikandan et al. 2015, Georgieva and Hlebarska 2017)
Ascomycota	*Botryosphaeria dothidea*	pathogenic	(Garcia, Aguirre et al. 2017)
Ascomycota	*Sclerotinia sp. 5/97-18*	unknown	(Brodal, Warmington et al. 2017)
Ascomycota	*Pestalotiopsis microspora*	controversial	(Strobel, Ford et al. 2002)
Ascomycota	*Myrothecium atroviride*	unknown	
Basidiomycota	*Rhodosporidium diobovatum*	beneficial	(Utkhede and Koch 2004)
unclassified	*Umbilicaria lyngei*	unknown	
Basidiomycota	*Rhodotorula nothofagi*	unknown	(Cornelissen, Botha et al. 2003)
Basidiomycota	*Kockovaella sacchari*	unknown	
Ascomycota	*Umbilicaria decussata*	unknown	(Beyer, Bölter et al. 2000)
unclassified	*Septoriella phragmitis*	unknown	(Van Ryckegem and Verbeken 2005)
unclassified	*Rhodotorula minuta*	beneficial	(Patiño‐Vera, Jimenez et al. 2005)
Ascomycota	*Colletotrichum lagenaria*	unknown	
Ascomycota	*Candida rancensis*	unknown	(Ramírez and González 1984)
Ascomycota	*Leohumicola minima*	unknown	(Hirose, Hobara et al. 2017)
Basidiomycota	*Pseudofavolus cucullatus*	controversial	(Yuan, Ji et al. 2017)
Ascomycota	*Diaporthe rudis*	pathogenic	(Torres, Camps et al. 2016)
unclassified	*Rhodosporidium sphaerocarpum*	unknown	(Butinar, Santos et al. 2005)
Ascomycota	*Peyronellaea glomerata*	controversial	(Deng, Paul et al. 2011)
Ascomycota	*Thermomyces lanuginosus*	unknown	(Singh, Madlala et al. 2003)
unclassified	*Cystofilobasidium infirmominiatum*	Beneficial	(Liu, Wisniewski et al. 2011)

Ascomycota	*Periconia macrospinosa*	controversial	(Mandyam, Fox et al. 2012)

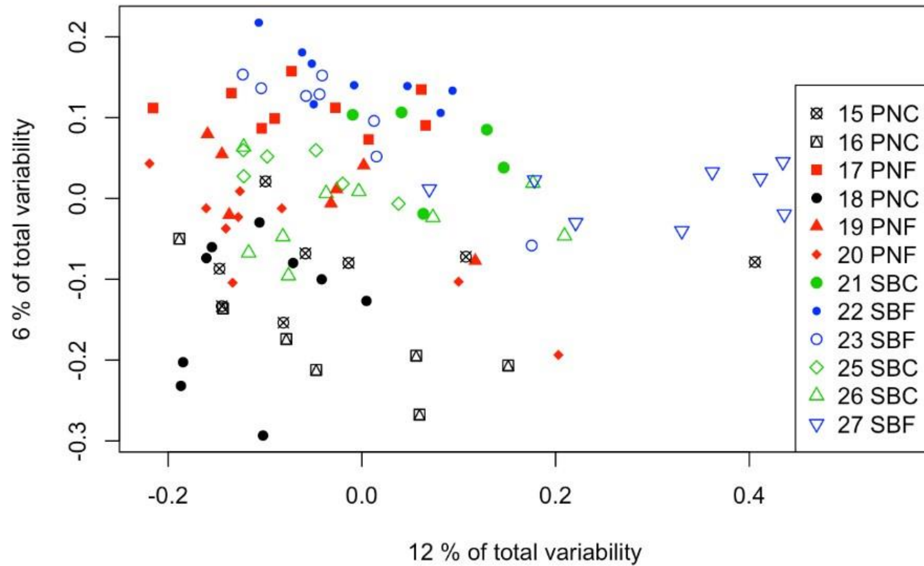
2.3.3 General similarity of selected VE loci: OTUs, GTD symptoms, management systems and grapevine varieties

We established statistics model to analysis the general similarity of microbiome among all vineyards for a general understanding variation of fungal OTUs in vineyards. All chosen loci were divided into four groups to show on the picture: Pinot Noir under contemporary management (PNC); Pinot Noir under future management (PNF); Sauvignon blanc under contemporary management (SBC) and Sauvignon blanc under future management (SBF).

The first approach studied two principal coordinates based on Bray-Curtis distances between quarter-root transformed counts. This approach means that common zeros (two sites both missing the same fungus) do not increase similarity (Figure 2.1). The distance between every two loci represents the total OTU variability with a short distance representing a high similarity overall.

These figures (Figure 2.1) suggest that there was no significant tendency in symptoms regarding cultivars or managing systems. However, some difference could still be found. : E.g. Vineyard 27 SBF locus tends to have long-distance from 18 PNC and 16 PNC. Cultivars of Pinot noir and Sauvignon blanc slightly influenced grapevine microbiome.

There was no obvious difference among cultivars and management found regarding symptomatic loci (Figure 2.1). For instance, 23 SBF has five symptomatic vines and 15 PNC contains four symptomatic vines.



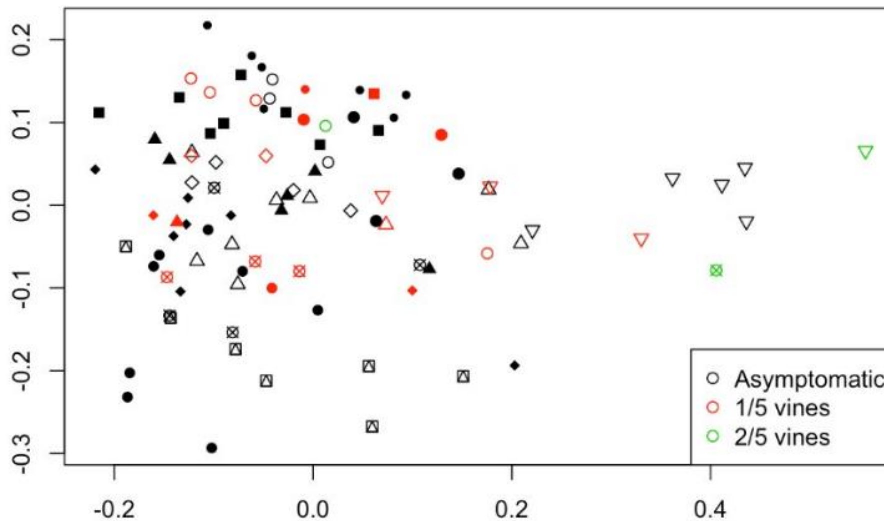


Figure 2.1. Principal coordinates using Bray Curtis on the count. Symbols represent the vineyard, while colours represent the grape variety or management in the first figure. The numbers of symptomatic vines are displayed in the lower figure.

2.3.4 Analysis factors affecting the proportion of *A. pullulans*, *E. lata* and GTD symptoms

Research questions and conclusions analysed by Beatrix Jones on fungal incidence and trunk symptoms in section 2.2.4 are listed below.

Question 1: Does variety and/or management and/or year affect *A. pullulans* abundance, treating vineyard location as a random effect?

Conclusion: Both management and variety affect the proportion of *A. pullulans*. Future managed vines have lower abundance of *A. pullulans* than contemporary managed vines. Sauvignon Blanc vineyards have lower *A. pullulans* abundance than Pinot noir vineyards.

Question 2: Is *E. lata* affected by management or variety, while accounting for vineyard location effects?

Conclusion: *E. lata* is not affected by management but affected by variety: Sauvignon Blanc vineyards have more *E. lata* than Pinot noir vineyards.

Question 3: Is *E. lata* abundance associated with *A. pullulans* abundance, after controlling for vineyard location, management and variety?

Conclusion: Yes, there is evidence of an association with *A. pullulans* reducing the abundance and proportion of *E. lata*.

Question 4: Is the presence of symptoms influenced by management or variety, after controlling for vineyard location?

Conclusion: No, the presence of trunk symptoms is not influenced by variety or management.

Questions 5: Do the abundances of *E. lata*, *A. pullulans* and their interaction predict the presence of symptoms, controlling for vineyard location?

Conclusion: *E. lata* and *A. pullulans* have no association with symptoms, either separately or as a combination with the current statistical model.

More detailed statistical results are in Appendix 3: (Data analysis 1 and Data analysis 2).

2.3.5 Associate *E. lata* and *A. pullulans* biomass with GTD symptoms

The interaction between fungal abundance (*A. pullulans* and *E. lata*) and GTD symptoms did not fully correspond to our predictions. There was no statistical support that *A. pullulans* can protect vines or *E. lata* harm on vines (Figure 2.2). Many canes with high *E. lata* abundance showed no GTD symptoms by the time when visual observation was made

(Question 3 and 4, section 2.3.4). At least from the results of our current statistic model, there is no evidence *A. pullulans* can reduce GTD symptoms.

We tried to visualize the relationships with the following, taking advantage of the fact that *A. pullulans* is shown in all loci and can be transformed into a log model (Y-axis of Figure 2.2). There are three symptomatic loci with no *E. lata* identified (No *E. lata*, 1/5 with symptoms, Figure 2.2).

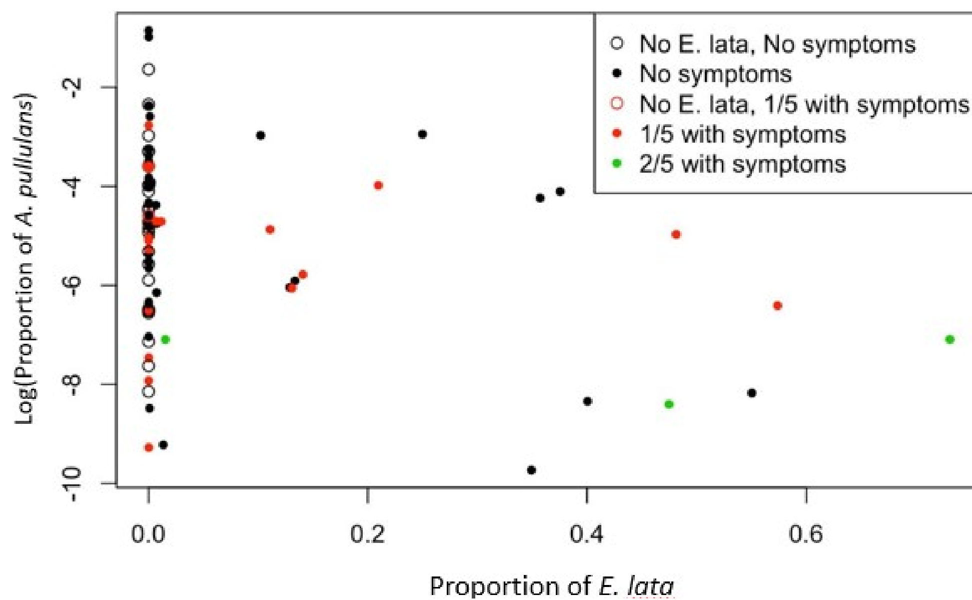


Figure 2.2. Loci GTD status with the proportion of *Eutypa lata* and *Aureobasidium pullulans* as axes. Colours and blank represent GTD symptoms of loci. X-axis and Y-axis represent proportion of *E. lata* and log proportion of *A. pullulans* in total fungal OTU reading, respectively.

2.3.6 Factors affecting GTD symptoms and fungal abundance

2.3.6.1 Associations between symptoms and profile of *A. pullulans* and *E. lata*, variety, year and management

At first, we intended to determine how *A. pullulans* and *E. lata* might affect the overall microbiome by combining data analysis with PCR for the detection of the two fungi. However, *A. pullulans* was detected in all five-vine-composites according to our NGS data. Thus, PCR detection that would provide 'presence/absence' information would be insufficient to link the two fungi with symptoms. Instead, a molecular method that required quantification of amounts of fungi present was required, i.e. real-time PCR.

A generalized linear mixed model was fit for variables, with transformed fungal levels to relevant *A. pullulans* and *E. lata* with GTD symptoms. Unexpectedly, none of the Year, Variety or management was significantly linked to the symptoms of each locus. Therefore, associations between symptoms and the fungal profile were considered. The effects on the fungal profile of Year, Management and Variety were controlled for by stratifying using vineyard and year. Individual associations with *E. lata* or *A. pullulans* were also insignificant. We expected levels of these two fungi were influenced by at least one variable, but from statistical analysis of the 2016/17 and 2017/18 data, we cannot make such a claim.

Grapevine GTD symptoms were not affected by Year, Management, Variety and *A. pullulans*. But symptoms were significantly associated with the transformed abundance proportion of *E. lata*, in another way; *E. lata* is a strong predictor of grapevine symptoms (Figure 2.3).

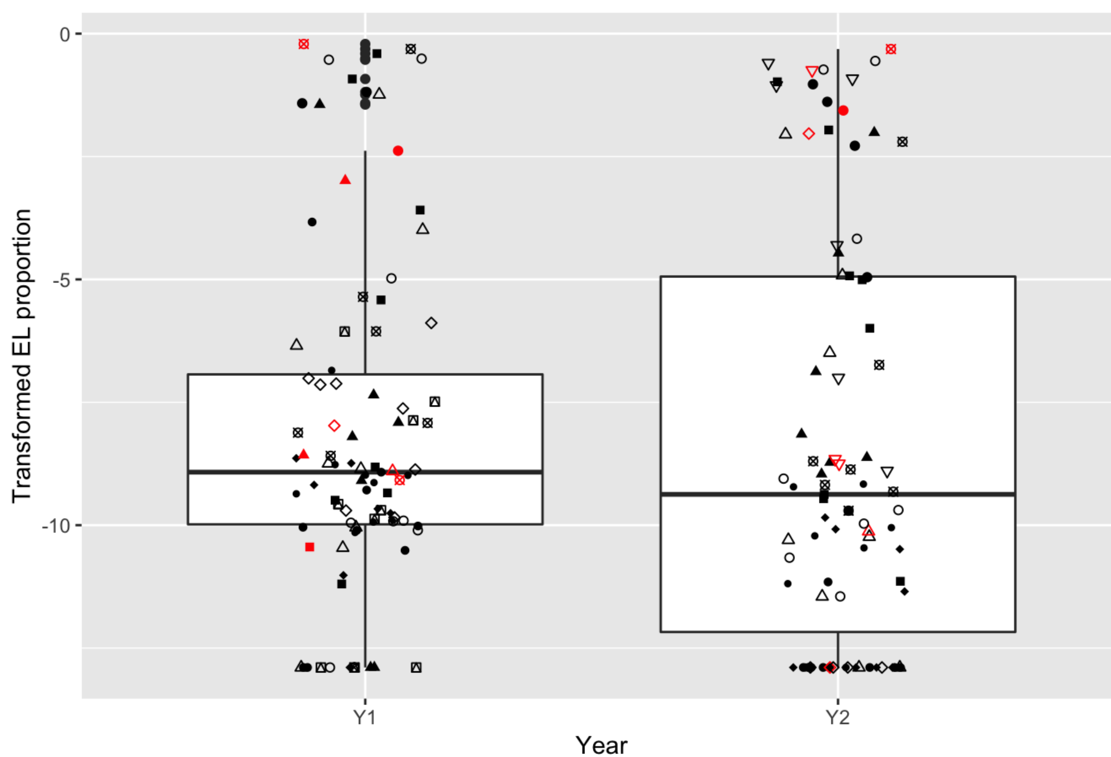


Figure 2.3. Transformed *Eutypa lata* levels and refined symptoms across two years. Symbols show vineyards, red indicates the presence of at least one symptoms.

From loci analysis results of GTD symptoms and fungal proportion of *A. pullulans* and *E. lata*, 13 loci of particular interest were identified (Figure 2.4). Within all five-vine-composites, one

specific locus had a low proportion or no *E. lata* but was identified with GTD symptoms (Figure 2.4, locus 7). At the same time, some loci shared a similar proportion of *A. pullulans* and *E. lata* respectively but displayed different GTD symptoms (Figure 2.4, loci 4, 5, 6, 10, 11, 12). Another locus had relatively high *E. lata* and low *A. pullulans* but no symptoms (Figure 2.4, locus 13). Several loci had moderate levels of both fungi and symptoms (Figure 2.4, loci 1, 2, 3, 8, 9).

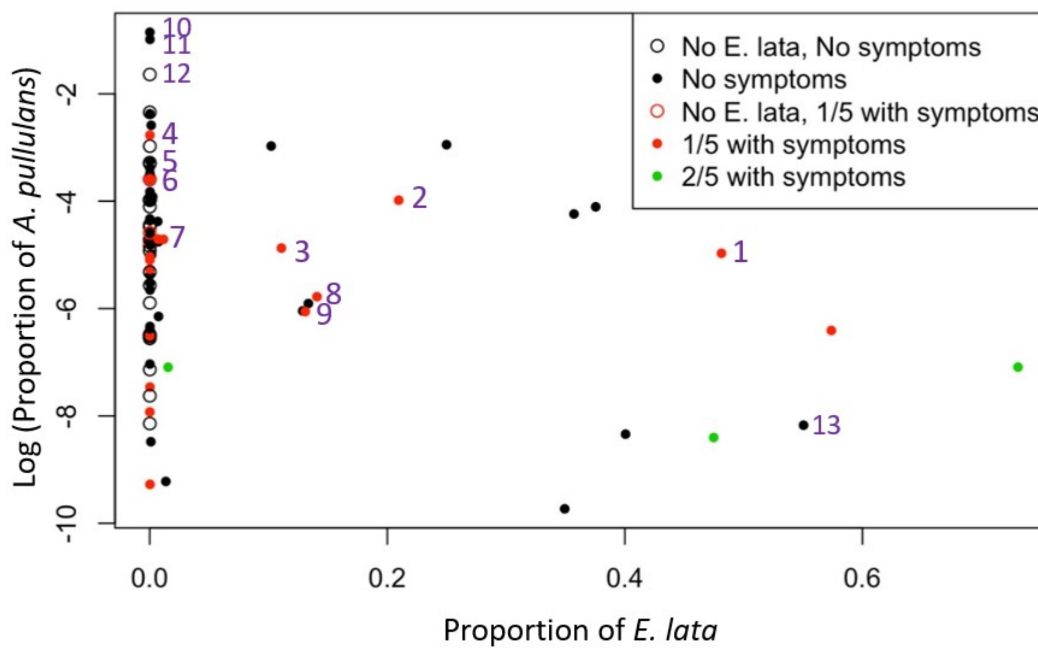


Figure 2.4. Loci GTD status with the proportion of *Aureobasidium pullulans* and *Eutypa lata*. Each dot represents a locus and colour represents the status of GTD symptom in locus. Numbers to the right of some dots represent codes of specific loci to be quantified in future.

2.4 Discussion

2.4.1 Summary of results

This chapter addressed two hypothesises in project aim 1 (Chapter 1.6).

The first hypothesis is that "The presence of *A. pullulans* in grapevines reduces the abundance of *E. lata* and decreases GTD symptoms". When addressing this hypothesis, this study discovered no significant relationship between the sequence abundance of *A. pullulans* and *E. lata* at a loci level in VE vineyards. However, *A. pullulans* was associated with decreased GTD symptoms and expressed weak protective effects according to the *in silico* analysis (Section 2.3.4).

The second hypothesis, "Vineyard variables (grapevine variety, management methods, year and location) impact on the abundance or severity of *A. pullulans*, *E. lata* and/or GTD symptoms", found that the abundance of *A. pullulans* is more sensitive to vineyard variables than *E. lata* (Section 2.3.4).

2.4.2 Statistical analysis of fungal NGS data and grapevine trunk symptoms

A linear mixed model was established to describe the effect of grapevine variety and management on both *A. pullulans* and *E. lata* when controlling for vineyard effects. Also, a potential association between two fungi when controlling for other conditions were analysed. A transformed format of NGS data helped to reduce inconsistency of fungal counts in all grapevines.

Vineyards under both future and contemporary management were assessed in this chapter. Although future managed vines had lower abundance of *A. pullulans* than contemporary managed vines (Q1, section 2.3.4), the presence of trunk symptoms is not influenced by variety or management (Q4, section2.3.4). Ideally, management methods would have played an important role in ruling fungal OTUs and GTD symptoms, however, this was not the case in the data set analysed. In order to provide growers with accurate and predictive suggestions, a more extensive comparison must be made. If results uncovered a promising consequence with future management, it would underpin a message to growers to change their current growing methods. Alternatively, improved management options under the

"future management" mandate could be developed and tested to achieve better GTD outcomes and a more efficient system.

2.2.1.1 GTD symptoms recording in vineyards

The VE project is a long-term research programme which requires data collection and analyse for five continuous years. Within one year, grapevines often start to express foliar symptoms from mid-spring: late October till November onwards. Trunk sampling and NGS sequence required a large amount of manual effort and are time-consuming. The symptoms and NGS data in this study were based on recording in year 2017-2018, however further data has subsequently become available to test the rigour and progression of the correlations identified in this chapter.

2.4.2.1 Impact of *A. pullulans* and *E. lata* on vine symptoms:

At least from results of this study to date, neither *A. pullulans* nor *E. lata* had an impact on GTD symptoms with support of a statistical significance (Q5, section 2.3.4). Even we found no direct link between fungal abundance and GTD symptoms, we can still have meaningful explanations based on our findings of Chapter 2.

The existing NGS data reveals the existence of *A. pullulans* in all five-vine composites, however its abundance varies. *E. lata*, by contrast, was identified in only a limited proportion of vines among vineyards. The inconsistency of *E. lata* in vines may eventually indicate a significant impact on grapevine health, although to date only a low ratio of tested vines were recorded as symptomatic. Moreover, most unhealthy vines expressed only one or two symptoms and this may have reduced the significance of the fungal impacts. For instance, results would be more representative if *E. lata* was identified in more vines or more vines were symptomatic.

Regarding trunk symptoms collection, a challenging task is to define whether a specific grapevine is "symptomatic" or "asymptomatic", and this difficulty may significantly affect the statistical analysis and results from this analysis. Even an experienced examiner could not completely avoid 'calling' false-positive results. For instance, symptoms such as "stunted shoots" or "small leaves" can easily be overlooked when the examiner walks along the rows. In particular, it is especially difficult to distinguish "young and newly grown" shoots or leaves

with those with minor symptoms. The disease rate in fact may be higher than our current records.

A longer study period will benefit the research described here and the goals of the VE project. In fact, the VE project extends through five years of research on these vineyards, which should provide sufficient information to study the development of fungal component and trunk symptoms using the methods described here.

One shortcoming of the data analysed here is that within the studies vineyards the GTD rates are diverse and can be very low in specific loci. A large proportion of grapevines showed no GTD symptoms even though the five vine-composite was identified with a high abundance of *E. lata*. These facts demonstrate that there is still much we do not understand about *E. lata* and GTD development.

Microbial communities often affect organisms' defense system and thus play the roles to reduce the burden of pathogens (Flint, Duncan et al. 2007). Recent research discovers about the relationship of bacteria microbiota in human nose/throat and susceptibility to influenza infection may be a useful analogy (Lee, Gordon et al. 2019). Despite different study subjects (humans and grapevines), our study used a similar analysis for individual 'oligotypes' (to identify closely related by distinct micro-organisms in HTS of the fungal gene). Using a more simple approach than Lee and colleagues, we only assessed an association with symptoms, controlling for vineyard effects (equivalent to their household effects), and no other covariates (they have age, smoker in household, etc as variables and used a screening technique to select potentially important ones). Future analysis of GTDs may include additional co-variates in the future.

2.4.2.2 Interaction of two fungi in NGS data

As hypothesised, the presence of *A. pullulans* can reduce the abundance of *E. lata* but failed to reduce GTD symptoms (Q5, section 2.3.4). This unexpected phenomenon indicates that the mechanism behind fungal interaction is more complicated than our hypotheses. Apart from direct antagonism between *A. pullulans* and *E. lata* and triggered plant defense effect, other BCAs or pathogens may also influence overall GTD symptoms. In the future, as we understand the component and their interactions better it will be important to consider the grapevine

and its entire microbiome as an ecosystem. In the meantime, performing more simple interaction tests *in vitro* and *in planta* would be appropriate to understand some of the contributing interactions that have been analysed together in this chapter. In the following chapters, we attempt to achieve a better understanding of the interactions between the two fungal species and their impact on grapevines.

2.4.4 Limitation of two management systems and future improvement

2.4.4.1 Influence of management systems on grape and soil microbiome

The analysis in this chapter revealed that the microbiome was influenced effectively by management systems and location (Q1, section 2.3.4). Future managed vines have a lower abundance of *A. pullulans* than contemporary managed vines, however, we did not notice a significant change in the disease rate or pathogen existence in future managed vineyards (Q2 and Q4, section 2.3.4). Analysis of additional years of data may clarify the correlations of these variables. Ideally, improved health status was predicted to be found in future managed vineyards and this helps to demonstrate the importance of developing current management systems.

Limited herbicide was applied in future managed vineyards to maintain under-vine cover and reduce the application of synthesized chemicals. Apart from affecting plant species around grapevines, the use of herbicide also has impacts on soil microbial pool but there is no evidence it contributes to fruit yields and quality (Chou, Heuvel et al. 2018). Other vineyard management or environmental factors such as temperature, location and grape cultivar have a stronger impact on grapevine microbiome in other studies (Bokulich, Thorngate et al. 2014). Some known microbial dispersal mechanisms can transport microbes from the ground to crops like wind, rainfall and insect activities (Madden 1997, Bock, Cook et al. 2012). However our results rejected our initial hypothesis that future management practice contributes to different grapevine microbiome and protects grapevine from trunk diseases.

This study does not focus on bacterial components, but research suggests that glyphosate (a common herbicide in vineyards) affect bacteria and fungi differently despite some inconsistent results (Araújo, Monteiro et al. 2003, Ratcliff, Busse et al. 2006, Weaver, Krutz et

al. 2007). Some studies recorded an increased soil CO_2 respiration rate and minor changes in bacterial community structures after application of glyphosate. An annual application of glyphosate decreased arbuscular mycorrhizal fungi (AMF) spores densities by 56% (Druille, García-Parisi et al. 2016). Intriguingly, to date our research result (Question 1, section 2.3.4) has revealed future managed vineyards has a lower abundance of *A. pullulans* than those under contemporary management (with annual glyphosate application). This result does not match some other studies that glyphosate can reduce *A. pullulans* radial growth and germination (Cheng 2015). There might be other herbicides playing roles or other fungal OTUs were more sensitive than *A. pullulans* to herbicides, that *A. pullulans* survived after "herbicide selection".

2.4.4.2 Future quantification of *A. pullulans* and *E. lata* biomass in selected VE loci

There are two hypotheses discussed in this section:

1. *E. lata* causes GTD symptoms or at least acts as a risk factor;
2. *A. pullulans* is protective against GTDs caused by *E. lata*.

For hypothesis 1, this study proved *E. lata* is a predictor of GTD symptoms by analysis. Also, hypothesis 2 can explain some cases where *E. lata* was present but the vines are asymptomatic. However the protective effect of *A. pullulans* was statistically weak (Q5, section 2.3.4). This negative response may be due to other mechanisms beyond *A. pullulans* reducing the abundance of *E. lata.*

From the analysis of loci, GTD symptoms and fungal proportion of *A. pullulans* and *E. lata* (section 2.3.6), 13 loci of particular interest were identified (Figure 2.6). Within some specific, the five-vine-composites had a low proportion or no *E. lata* identified but the vines displayed GTD symptoms. At the same time, some loci shared a similar proportion of *A. pullulans* and *E. lata* respectively but displayed differently in GTD symptoms (Figure 2.6). For those numbered VE loci (Figure 2.6), quantification of their *A. pullulans* and *E. lata* abundance could uncover more information about the interaction of the two fungi and GTD symptoms.

For these loci, quantification of fungal abundance at an individual vine level would help us understand the relationship fungi and GTD symptoms. Specifically, quantification of *A.*

pullulans and *E. lata* at the individual vine level would provide detailed fungal information to correlate with individual vine GTD symptoms. Information of Circled loci (Figure 2.4) were listed in Table (2.2).

Table 2.2. Information of 13 circled loci (Figure 2.4) with specific interest to run NGS in the future. High *A. pullulans* abundance represents Log (Proportion of *A. pullulans*) ≥ -4; High *E. lata* abundance represents Proportion of *E. lata* ≥ 0.2; 0 *E. lata* abundance represents *E. lata* was not identified in a locus.

Locus number	GTD symptom (0/1)	High *A. pullulans* (Yes/No)?	High *E. lata* (Yes/No/0)?	Grapevine variety	Management
1	1	No	Yes	Sauvignon Blanc	Future
2	1	Yes	Yes	Sauvignon Blanc	Contemporary
3	1	No	No	Pinot Noir	Contemporary
4	1	Yes	No	Pinot Noir	Contemporary
5	0	Yes	No	Pinot Noir	Contemporary
6	1	Yes	No	Pinot Noir	Future
7	1	No	No	Pinot Noir	Future
8	1	No	No	Pinot Noir	Future
9	1	No	No	Sauvignon Blanc	Contemporary
10	0	Yes	No	Sauvignon Blanc	Contemporary
11	0	Yes	No	Pinot Noir	Future
12	0	Yes	0	Sauvignon Blanc	Contemporary
13	0	No	Yes	Sauvignon Blanc	Future

These identified loci include five-vine samples that comprise symptomatic plants with high *E. lata* and low *A. pullulans* (Figure 2.4, loci 8 and 9); asymptomatic loci with *E. lata* but high *A. pullulans* (loci 10 and 11); and asymptomatic loci without *E. lata* (loci 12). There are also points that do not fit our hypotheses: asymptomatic loci with high *E. lata* (locus 13); symptomatic loci with *E. lata* and high *A. pullulans* (loci 1-3: high *E. lata* and loci 4, 5, 7: low *E. lata*); symptomatic points without *E. lata*: (loci 12).

Due to lack of fungal OTU information at an individual vine level, the abundance of *A. pullulans* and *E. lata* remains unknown for each vine, therefore, we could not yet link the exact fungal microbiome with GTD symptoms directly. Since section 2.4.1 showed that a quantitative rather than a qualitative (that reveals only presence or absence) assessment would be required to distinguish significant changes in the abundance of *A. pullulans* and *E. lata*, real-

time PCR could be performed to individual grapevine DNA in these 13 identified loci. DNA of individual grapevines from these loci has been received and quantification of two fungal DNA could be undertaken in the near future. Unfortunately, there was insufficient time and additional expenses associated with this analysis and it could not be completed during the thesis research period. This process will require the use of reference genes to perform quantitative real-time PCR (Brinkhof, Spee et al. 2006).

3. Is *Aureobasidium pullulans* antagonistic to *Eutypa lata in vitro?*

3.1 Introduction

As described in Chapter 2, *A. pullulans* or *E. lata* were not significant indicators of GTD symptoms in vineyards, however, *A. pullulans* was associated with a low abundance of *E. lata*. *E. lata* is one of the principal causal agents of Eutypa die-back (Section 1.3) and is often a focus in GTD research (Ferreira, Matthee et al. 1991, Rolshausen, Mahoney et al. 2006, Halleen, Fourie et al. 2016, Pouzoulet, Rolshausen et al. 2017). By contrast, *A. pullulans* has previously been demonstrated to have inhibitory effects on multiple fungal phytopathogens (Ippolito, El Ghaouth et al. 2000). Therefore, further experiments performed on these two fungi may clarify whether *A. pullulans* is actively acting to control the proliferation of *E. lata* in commercial vineyards within New Zealand.

Understanding how *A. pullulans* may affect *E. lata* growth *in vitro* is a direct approach to determine any potential biocontrol activity. The *in vitro* method uses purified isolates of each fungus and determines the colony radius and calculated inhibition rate of *E. lata* by four *A. pullulans* strains. Inhibition of *E. lata* in vitro may by indicate biocontrol activity of *A. pullulan*, however, further evidence would then need to be conducted *in planta* to determine the BCA efficacy within a host (including the plant defense system and physiological structures) and in a naturally full microbiome ecosystem.

To address Research Aim 2, "to identify the strain of *A. pullulans* that provides the best biocontrol of *E. lata* within *in vitro* conditions", this chapter applied single or dual fungal colony culturing on Potato Dextrose Agar (PDA) plates as was performed in section 3.2. From a similar study on *A. pullulans* and *E. lata*, *in vitro* study was performed on PDA plates with measuring colony growth status of colonies (Don, Schmidtke et al. 2019). We also performed single or dual dual-culturing of *E. lata* and *B. cinerea* on PDA plates with the same protocols to illustrate how a non-BCA might affect *E. lata* growth *in vitro*. The flow chart in Figure 3.1 illustrates the study methods in this chapter.

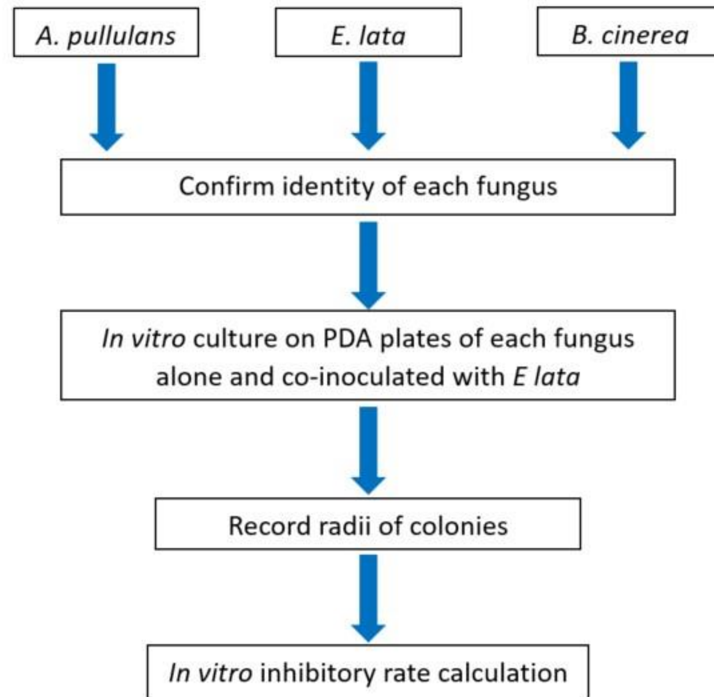


Figure 3.1. Flow chart to test for inhibition of *Aureobasidium pullulans* of *Eutypa lata*, compare with *Botrytis cinerea* and *Eutypa lata in vitro*. The workflow shows the steps to determine any *in vitro* antagonistic effects of *A. pullulans* compared with *B. cinerea* on *E. lata* growth.

3.2 Materials and methods

3.2.1 *A. pullulans*, *E. lata* and *Botrytis cinerea* strains used in the study

All strains were purchased from the International Collection of Micro-organisms from Plants (ICMP) of Landcare Research, Auckland, New Zealand, including four strains of *A. pullulans* and one strain of *E. lata* (Table 3.1). All strains were delivered in solid PDA cultures. Dion Mundy (Scientist, PFR Blenheim) first isolated and donated *E. lata* 20026 to the ICMP. An isolate of *B. cinerea* (wild-type, isolated from Sauvignon blanc grapevines in Pukekohe, Auckland) was kindly provided by Kai Lewis (Research Associate, PFR Auckland).

All strains of *A. pullulans* and *E. lata* were cultured on PDA medium for both *in vitro* (Chapter 3) and *in planta* (Chapter 4) tests. Before use, pure cultures were maintained at room temperature in the dark for 7 days. For pure fungal DNA extraction, all *A. pullulans* and *E. lata* strains were cultured in Potato Dextrose Broth (PDB, Becton, Dickinson and Company, France) liquid culture and centrifuged.

Table 3.1. Strains of *Aureobasidium pullulans, Eutypa lata* and *Botrytis cinerea* used in this research. *A. pullulans* and *E. lata* isolates were purchased from Landcare Research, Auckland, *B. cinerea* isolate was provided by Kai Lewis at PFR Auckland.

Species	ICMP Barcode	Origin species	Origin tissue	Region
A. pullulans	ICMP 3057	*Malus domestica*	leaf	Auckland
A. pullulans	ICMP 21143	*Dodonaea viscoca*	leaf	Canterbury
A. pullulans	ICMP 19713	*Cytisus scoparius*	stem	Canterbury
A. pullulans	ICMP 20345	*Ranunculus acris*	stem	Nelson
E. lata	ICMP 20026	*Vitis vinifera*	grapevine trunk	Marlborough
B. cinerea	Wildtype	*Vitis vinifera*	grapevine trunk	Auckland

3.2.2 Fungal DNA extraction from pure cultures

Pure fungal cultures were purified either using a commercial kit (3.2.2.1) or the cetyl trimethylammonium bromide (CTAB) method (3.2.2.2). Extracted DNA was used as a template for all end-point PCR and real-time PCR research within Chapters 3 and 4.

3.2.2.1 Fungal DNA extraction by DNeasy® Kit

Pure fungal cultures scraped from PDA plates was used for DNA extraction using the DNeasy® Plant Mini Kit (QIAGEN) according to the protocol of the manufacturer. Purified DNA was

assessed by DS-11 Fluorometer (DeNovix, USA) for yield and quality by testing OD260 and OD280 value.

3.2.2.2 Fungal DNA extraction method by CTAB

After extraction fungal DNA by DNeasy® Kit, we also applied CTAB method to extract pure fungal DNA. Method protocols were kindly provided by Dion Mundy and Rebecca Woolley (PFR, Blenheim). Pure fungal samples from overnight growth (25 mL) were collected by centrifugation at 8750x g for 20 min in an RC 6 Plus Centrifuge (Thermo Scientific, USA). The pellet resuspended in 900 µL CTAB buffer was incubated for 90 min at 65 °C with vortexing every 15 min, then all samples were centrifuged at 15,000x g for 5 min and 500 µL liquid was removed for the following steps. An equal volume (500 µL) chloroform: isoamyl alcohol (24:1) was added and thoroughly vortexed then centrifuged for 10 min at 15,000x g. The upper aqueous phase (normally 400 µL) was carefully removed without disturbing other layers and transferred to a new labelled tube. Subsequently, 0.08 volumes of cold 7.5M ammonium acetate and 0.54 volumes of cold isopropanol was added and mixed gently by inverting each tube a few times prior to chilling at -20°C overnight then centrifuging at 15,000x g. for 30 min. The supernatant was discarded and the pellet washed with 700 µL ice-cold 70% ethanol followed by inversion of the tube gently to remove the supernatant without dislodging the pellet and air drying on a clean paper towel for 15 min. Finally, the pellet was dissolved in 40 µL UltraPure Distilled water (Invitrogen, USA) by incubating at 37°C for 30 min.

After extraction, DNA concentration and quality were measured by spectrophotometric quantification: DS-11 Fluorometer (DeNovix, USA).

3.2.3 Confirmation of *A. pullulans* and *E. lata* strains

A. pullulans (four strains) and *E. lata* were cultured onto PDA plates for morphological identification of colonies. Cultures were placed in Klip IT™ box (Sistema, New Zealand) at room temperature. The colony morphology was observed at the same time of day until colonies fully occupied the entire petri dish.

Each *A. pullulans* (four strains) and *E. lata* strains were subject to DNA isolation and end-point PCR to confirm their identity. Primers and protocols for end-point PCR are described in section 3.2.3.1.

3.2.3.1 Primers for PCR and real-time PCR

Primers for amplification of four *A. pullulans* strains and *E. lata* 20026 were selected from published articles and used for all end-point PCR and real-time PCR in this study (Table 3.2). Primers were purchased from Sigma-Aldrich New Zealand.

Table 3.2. Primers for amplification of *Aureobasidium pullulans* and *Eutypa lata* 20026 by end-point PCR and real-time PCR in this study.

Fungal species	Primer name	Sequence	Reference
A. pullulans	Forward: AP19F	5'-TACGGTGAAGCTGCGTGATGGCT-3'	(Chan, Puad et al. 2011, Pinto, Custodio et al. 2018)
	Reverse: AP386R	5'-TGGGTAATTTGCGCGCCTGCT-3'	
E. lata	Forward: EIQF	5'-GCCAGCTAATAAAACAATTGCTTACCT-3'	(Pouzoulet, Rolshausen et al. 2017)
	Reverse: EIQR	5'-AGATAACCTCGTGTGATTGTGTGATT-3'	

3.2.3.2 End-point PCR for *A. pullulans* and *E. lata*

End-point PCR for species confirmation comprised the components outlined in Table 3.3. All reactions used Invitrogen Platinum Taq DNA Polymerase (Thermo Fisher Scientific, USA). For all PCRs of *A. pullulans* and *E. lata*, one positive control of pure fungal DNA from the target species and two negative controls (water instead of DNA template or pure DNA of the other target fungal species) were added.

Table 3.3. Contents for endpoint PCR of *Aureobasidium pullulans* and *Eutypa lata* DNA in confirmation of strains. Volumes listed are for a total reaction volume of 10 µL**.**

	Reagent	Concentration	Volume per reaction (µL)
Master mix	Water		6.6
	10 x DNA Buffer	10	1.0
	dNTPs	10 mM	0.3
	Magnesium	50 mM	0.5
	Forward primer	20 mM	0.2
	Reverse primer	20 mM	0.2
	Taq DNA polymerase	5 U/µL	0.2
Template	DNA or water	20 ng/µL	1.0

The PCR protocol with primers AP19F and AP386R for *A. pullulans* detection was: Initial denaturation at 94°C for 30 seconds, amplification for 40 cycles at 94°C for 10 seconds, 72°C for 40 seconds and a final extension at 72°C for 5 mins.

The protocols for *E. lata* detection with primers EIQF and EIQR was: Initial denaturation at 94 °C for 20 sec, followed by 40 cycles of 94°C for 3 seconds and 60°C for 30 seconds with a final extension at 60°C for 5 mins. All PCR reactions were performed in a TC-412 PCR machine (Techne, UK).

3.2.3.3 Gel electrophoresis for products of end-point PCR

Endpoint PCR products from both trial experiments and sensitivity tests were separated by gel electrophoresis to determine their size.

To each PCR sample, 1 µL loading dye (6 X) was added then mixed by pipetting and loaded on to either a 2% or 2.5% agarose gel (HydraGene, USA, made with 1 X TAE buffer) for *A. pullulans* or *E. lata*, respectively. Invitrogen 1 kb plus ladder (Thermo Fisher Scientific, USA) was also loaded was added with to determine the size of *A. pullulans* products and the 50 bp ladder was used for *E. lata* products.

Prior to electrophoresis, Invitrogen SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, USA) was added 1 µL to bind and visualise amplified DNA by UV after DNA separation. Electrophoresis was performed using 1 X TAE running buffer with a PowerPac Basic Power Supply (BIO-RAD, USA) for 30 mins at either 80 or 120 V for *A. pullulans* and *E. lata*, respectively.

3.2.3.4 Real-time PCR for *E. lata* strain confirmation

The real-time PCR protocol for *E. lata* was generated from a published article (Pouzoulet, Rolshausen et al. 2017). All real-time PCRs were performed with Eco™ Real-Tim PCR System (Illumina, USA) and related consumables. Real-time PCR exploited the SYBR-Green method for *E. lata* using the same primers EIQF and EIQR as used for real-time PCR (section 3.2.3.1).

The *E. lata* real-time PCR protocol comprised an initial denaturation at 95°C for 5 mins, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 64°C for 20 sec and extension at 72°C for 20 sec. An additional melting analysis of 95°C-55°C-95°C was applied at the end of PCR to generate melt curves. Each real-time reaction contained 6.8 µL water, 2.0 µL 5x FIREPol Master Mix (Solis BioDyne, Estonia), 0.1 µL EIQF (forward primer) and 0.1 µL EIQR (reverse primer).

3.2.4 *In vitro* antagonism test of *A. pullulans* against *E. lata*

Fungal strains (Table 3.1) were received from Landcare in a solid agar slope media and were individually transferred for sub-culture on DifcoTM PDA plates (prepared as described by the manufacturer). For initial growth recovery, squares (~2mm x 2mm) from each slope tube were removed using a sterilized scalpel and transferred onto the surface of a PDA plate. Inoculated plates were maintained at room temperature with 12 hours light, 12 hours dark. Plates were cultured until the fungi almost colonized the entire plate surface, typically 14-21 days. *In vitro* experiments were performed at New Zealand Institute of Plant and Food Research, Auckland.

3.2.4.1 Culture of *A. pullulans* and *E. lata* on plates

Fungal plugs (5 mm x 5 mm) of pure *A. pullulans* or *E. lata* solid culture were cut from rapidly growing cultures on PDA plates. Mycelium plugs were used to inoculate each fungal strain onto PDA plates (6 replicates per treatment) alone or in combination resulting in ten different treatments as outlined in Table 3.4. Each mycelial plug was placed on 1 cm from edge of an 85 mm diameter petri dish (Thermos Scientific, USA). For dual-cultures, the second mycelial plug or PDA plug was placed on the opposite side of the plate. *In vitro* samples were then incubated at room temperature in the dark, within a Physical Containment Level 2 (PC2) laboratory, PFR Mount Albert, Auckland. Fungal colonies were visually observed at the same time on day 1, day 2, day 6, day 8, day 9, day 10, day 11 and day 12. The radii (cm) of all colonies were measured once the dual inoculated fungal colonies had contacted each other at their growing fronts, i.e. 10 or 12 dpi. Experiments were carried out at room temperature, according to the thermometer and air conditioner controller in the lab, whereby the room temperature ranged from 21°C to 23°C.

Table 3.4. Single and dual *in vitro* culture of *Aureobasidium pullulans* (four strains) and *Eutypa lata* (strain 20026). PDA solid cultures were inoculated with either *A. pullulans* (one out of four strains) and one *E. lata* mycelium plug, or one fungal mycelium plug and one PDA medium plug, or two PDA medium plugs. Fungal plugs were placed 1 cm from the edge of the petri dish with another plug on its opposite side. Each treatment was replicated on six plates.

Treatment No.	*A. pullulans*	*E. lata* 20026
1	3057	+
2	19713	+
3	20345	+
4	21143	+
5	-	-
6	3057	-
7	19713	-
8	20345	-
9	21143	-
10	-	+

3.2.4.2 Fungal colony radius recording and data analysis

The growth of fungal colonies (as assessed by colony radius) in different combinations on a single plate was the principal study of the *in vitro* antagonism tests. When surface contact of

two colonies on the same plate was observed, a ruler was used to measure the radius of each colony on the line between the two inoculation points (Figure 3.2).

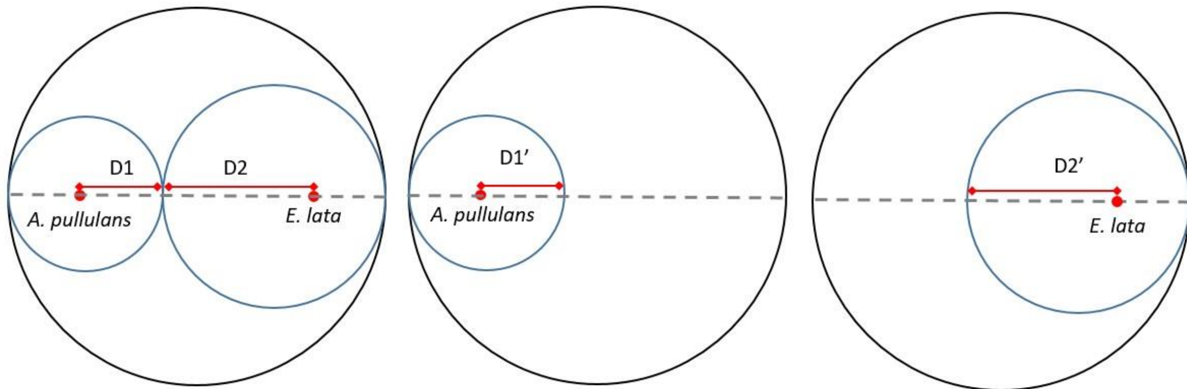


Figure 3.2. Measurement of fungal colony growth on a petri dish. The radius of each colony was measured from the fungal plug to the colony edge between the two plugs. Red spots represent inoculation points. Colony radii (D1 and D2) were measured and recorded.

Inhibition percentages were calculated by the formula. D1 and D1’ represent colony radii of the same *A. pullulans* strains grown on dual-culture and by itself, D2 and D2’ represent colony radii of *E. lata* grown on dual-culture and by itself.

$$I(inhibition\ percentage\ for\ A.pullulans) = \frac{(D1' - D1)}{D1\ '} \times 100\%$$

$$I(inhibition\ percentage\ for\ E.lata) = \frac{(D2' - D2)}{D2'} \times 100\%$$

One-Way ANOVA tests were performed to determine the significance of colony sizes among treatments. All calculations were performed at 95% confidence level of significance (Tang 2019).

3.2.4.3 Non-biological control agent culturing test: *Botrytis cinerea* and *Eutypa lata*

Fungal treatments included: *B. cinerea* cultured alone, *E. lata* cultured alone and dual-inoculations of *B. cinerea* and *E. lata*. Experiment methods were the same as section 3.2.3.1

and 3.2.3.2. The culturing and calculation methods followed the same as the antagonistic tests of *A. pullulans* and *E. lata* in sections 3.2.4.1.

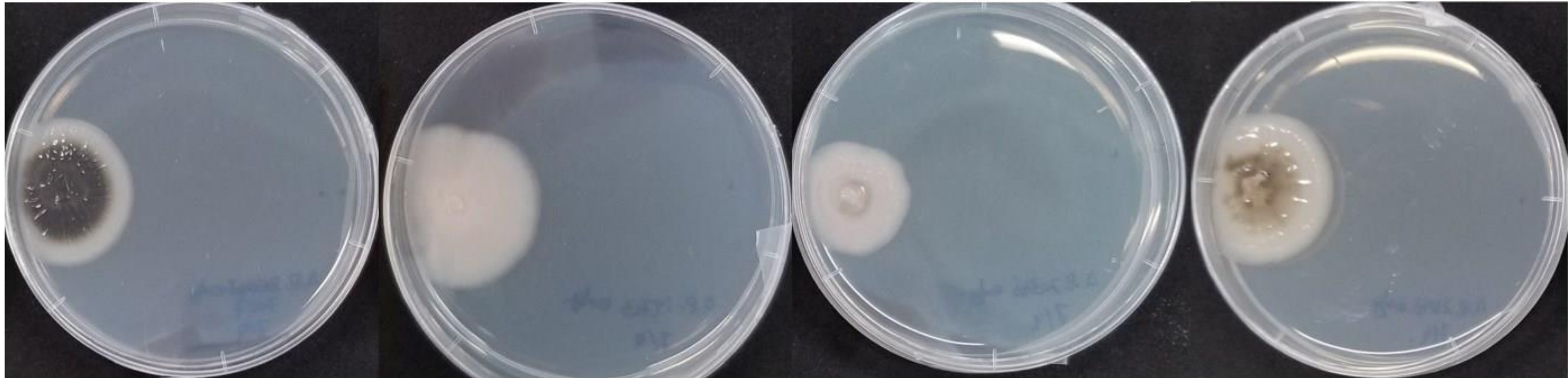
3.3 Results

3.3.1 Confirmation of the purchased fungal species

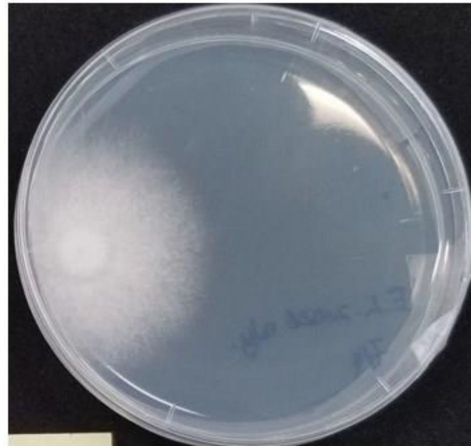
3.3.1.1 Morphological observation

Four *A. pullulans* expressed varied growth speed and pigmentation among strains, but shared similar edge and colony structures on PDA plates. *A. pullulans* 20345 grew slower than the other strains, while the growth rates of the remaining three strains were similar. All four *A. pullulans* strains formed circle-like colonies from their inoculation points with smooth surfaces with sharp and tidy colony edges. Pigmentation of colonies developed at different speeds. All four strains were at first white to light yellow colour and started to show darker pigments close to the inoculation points after one-week post inoculation. *A. pullulans* 3057 was the first strain to express dark green colour, i.e. within 6 dpi (Figure 3.3). At the same time, *A. pullulans* 19713 showed several concentric light brown circles while *A. pullulans* 20345 and *A. pullulans* 21143 had a larger proportion of brown colour than *A. pullulans* 19713.

Colonies of *E. lata* 20026 were morphologically distinct from those of *A. pullulans*. All replicates of *E. lata* 20026 were white in colour with fluffy and uneven colony edges (Figure 3.3). They had thin and obvious mycelial with varied radii to the colony margin.



A. pullulans 3057 | *A. pullulans* 19713 | *A. pullulans* 20345 | *A. pullulans* 21143

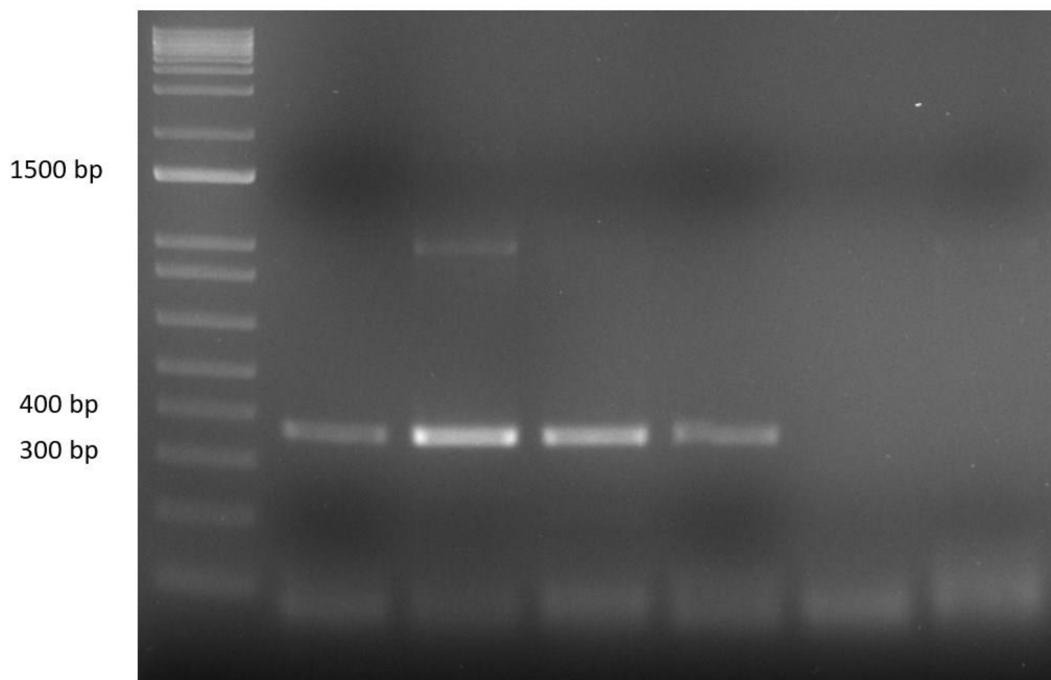


E. lata 20026

Figure 3.3. Colonies of *Aureobasidium pullulans* (four strains) and *Eutypa lata* 20026 on PDA plates (6 dpi). Four *A. pullulans* strains showed different colour but similar colony shape. *E. lata* colony was white and fluffy.

3.3.1.2 End-point PCR confirmation for *A. pullulans* and *E. lata* strains.

The end-point PCR method was applied to confirm the species of the purchased *A. pullulans* and *E. lata* isolates using the methods described in sections 3.2.2.1 to 3.2.3.3. Using the primers AP19F and AP386R and template DNA from the four strains of *A. pullulans* resulted in bands at the same size, near 400 bp, which was close to the expected size of 386 bp (Figure 3.4). Thus, all *A. pullulans* strains met the expectation and were considered to be the correct species.



A. pullulans strain:	3057	19713	20345	21143	-	-
E. lata 20026:	-	-	-	-	-	+

Figure 3.4. End-point PCR of four *Aureobasidium pullulans* strains with primers AP19F and AP386R. Each reaction contained 9 µL of master mix with 1 µL of template DNA from *A. pullulans* strain or *E. lata* 20026 or water as detailed below the gel image.

E. lata 20026 template DNA resulted in a band at the expected size at 126 bp, but this EIQF and EIQR primer pair also produced bands in the lanes templated by the *A. pullulans* DNA and water negative controls (Figure 3.5). Thus, we could not confirm that the purchased fungal isolate was indeed *E. lata*. After multiple attempts of changing each reagent and cleaning and changing the experimental equipment, the bands in the negative controls were not consistently absent as expected. Therefore the primer pairs EIQF and EIQR as used in

the end-point PCR was not sufficient to perform molecularly identify *E. lata* and further experiments with real-time PCR were undertaken.

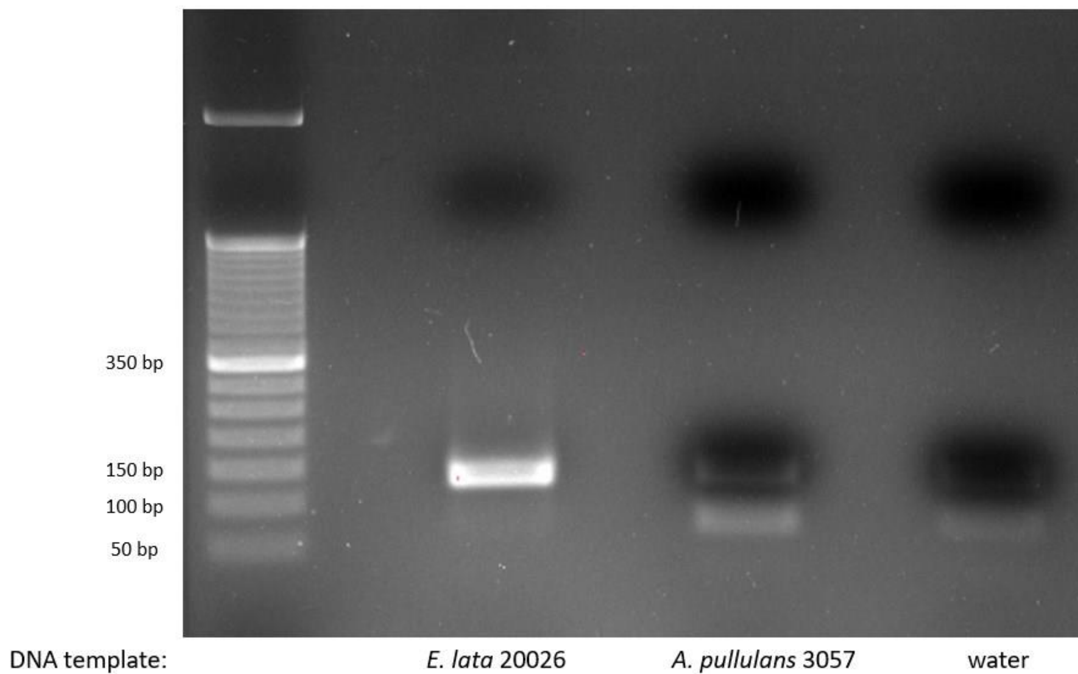


Figure 3.5. Endpoint PCR of *Eutypa lata* with primers EIQF and EIQR. DNA extracted from *E. lata* 20026 or *A. pullulans* 3057 were used as positive and negative controls, respectively, along with a water negative control. Either 1 µL of template DNA from *E. lata* 20026, *A. pullulans* strain 3057 or water was added in the reaction as detailed below the gel image.

3.3.1.3 Real-time PCR for strain confirmation of *E. lata* 20026

Further confirmation for *E. lata* with EIQF and EIQR was performed by real-time PCR of this section. To demonstrate primer specificity and *E. lata* species identification. Amplification curves revealed that real-time PCR can distinguish DNA of *E. lata* 20026 at 20 ng from *A. pullulans* 3057 at 20 ng (Figure 3.6) as there was a significant difference in the two Cq values (ΔCq>10) from the two DNA templates.

Melt curves performed after amplification illustrated that the *E. lata* and *A. pullulans* species were amplified with different products. *E. lata* had an obvious peak at Tm=82.6°C, while both water and *A. pullulans* shared peaks at Tm=77.2°C to 77.5°C (Figure 3.7). The combination of both real-time PCR and high-resolution melt curve analyses provides two convincing results that illustrate that the primer pair EIQF and EIQR specifically amplify *E. lata* 20026, the strain purchased for our study.

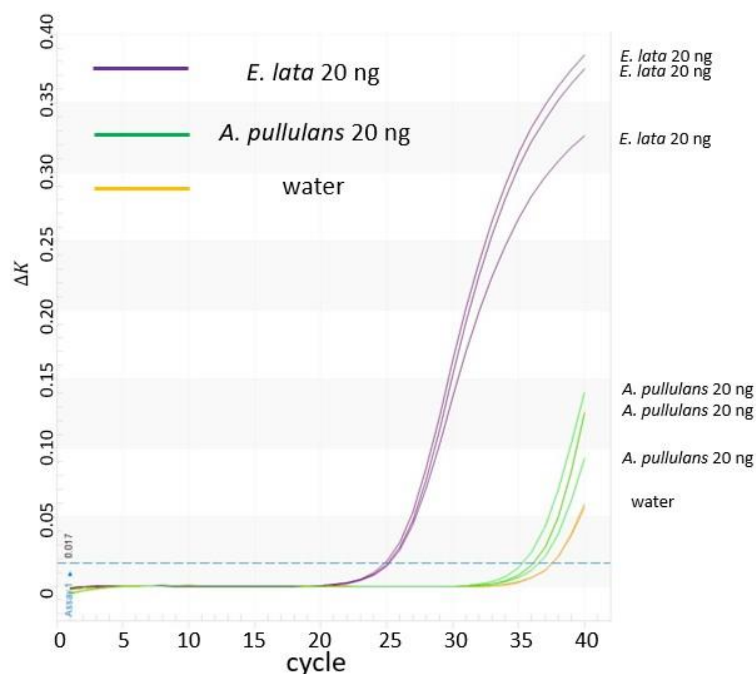


Figure 3.6. Amplification curves of *Eutypa lata* 20026 and *Aureobasidium pullulans* 3057 with primers EIQF and EIQR. Templates were differentiated by colours. *E. lata* (20 ng) was considered positively amplified while *A. pullulans* (20 ng) and water were considered negative.

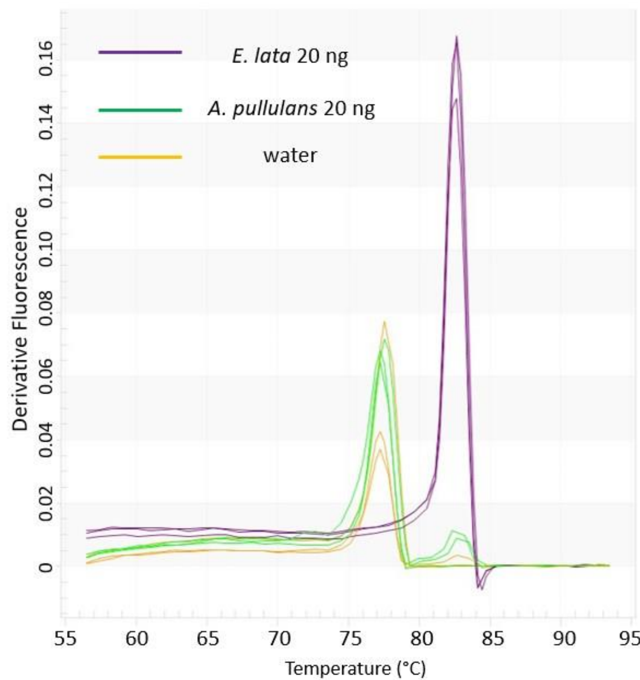


Figure 3.7. Melt curves of *Eutypa lata* 20026 (20 ng) and *Aureobasidium pullulans* 3057 (20 ng) with primers EIQF and EIQR. *E. lata* had reduction peak of fluorescence at 82.6°C, *A. pullulans* and water had a reduction peak at 77.2°C ~77.5°C.

3.3.2 Growth status of single and dual-inoculated *A. pullulans* and *E. lata* colonies

As outlined in Section 3.1, *A. pullulans* and *E. lata* were cultured alone or together on PDA medium for *in vitro* antagonism tests using the method described in Section 3.2.3. Colonies of co-inoculated *A. pullulans* and *E. lata* were shown in Figure 3.8.

Since all tests were replicated six times, and *A. pullulans* and *E. lata* colonies showed a variety of average radius in different fungal inoculations, the average colony size and variation within replicates was assessed (Figure 3.8). Detailed data of average colony radii and standard deviations are in Appendix 1.

3.3.2.1 *A. pullulans* alone and comparison between four strains

From day 1 to day 6, fungal colony sizes varied among the four *A. pullulans* strains when they were inoculated alone. *A. pullulans* strain 3057, 21143 and 19713 expressed similar growth rates while colony size of *A. pullulans* 20345 increased relatively slower than other the *A. pullulans* strains.

At day 10, colonies were measured for their radii and compared among treatments (Figure 3.9 and Appendix 1). When cultured alone, four strains of *A. pullulans* had average colony sizes from 1.85 cm to 2.55 cm. *A. pullulans* 20345 was the slowest-growing strain and all strains shared similar variabilities from 0.12 to 0.16. At day 12, *A. pullulans* 20345 reached an average radius at 2.36 cm with higher variability (Δ=0.20) than other strains.

All four strains of *A. pullulans* had reduced colony radii when cultured with *E. lata* on the same plate, compared to each corresponding *A. pullulans* strain cultured alone on PDA. Among all *A. pullulans* strains, *A. pullulans* 19713 showed the least colony reduction when cultured with *E. lata*; the average difference was 0.02 cm. By contrast, all other *A. pullulans* (3057, 20345 and 21143) had an average difference from 0.17 cm to 0.50 cm. *A. pullulans* 21143 was the most inhibited strain with a reduction of 0.50 cm.

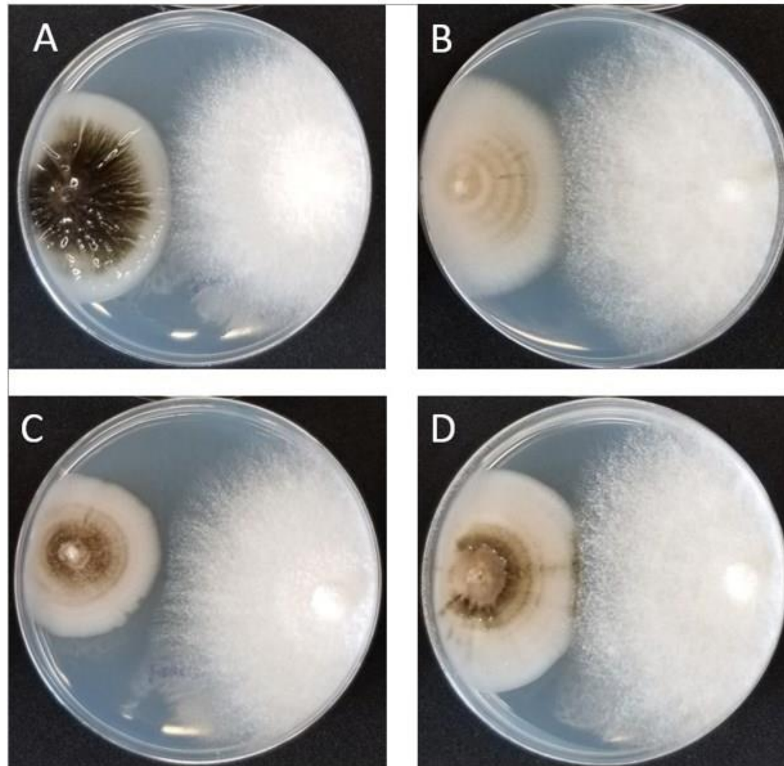


Figure 3.8. Pictures of *in vitro* antagonistic tests of *Aureobasidium pullulans* and *Eutypa lata* strains (10 dpi). Potato Dextrose Agar (PDA) were inoculated with pure fungal plugs (5 mm x 5 mm) of *A. pullulans* strain and *E. lata* from solid cultures (14 dpi). *A. pullulans* strains were inoculated on the left side of plates with *E. lata* opposite on the right. All *A. pullulans* strains except for *A. pullulans* 20345 (C) showed surface contact with *E. lata* colonies by 10 dpi. Co-inoculation with *A. pullulans* 20345 (C) resulted in a changed *E. lata* colony shape without direct contact.

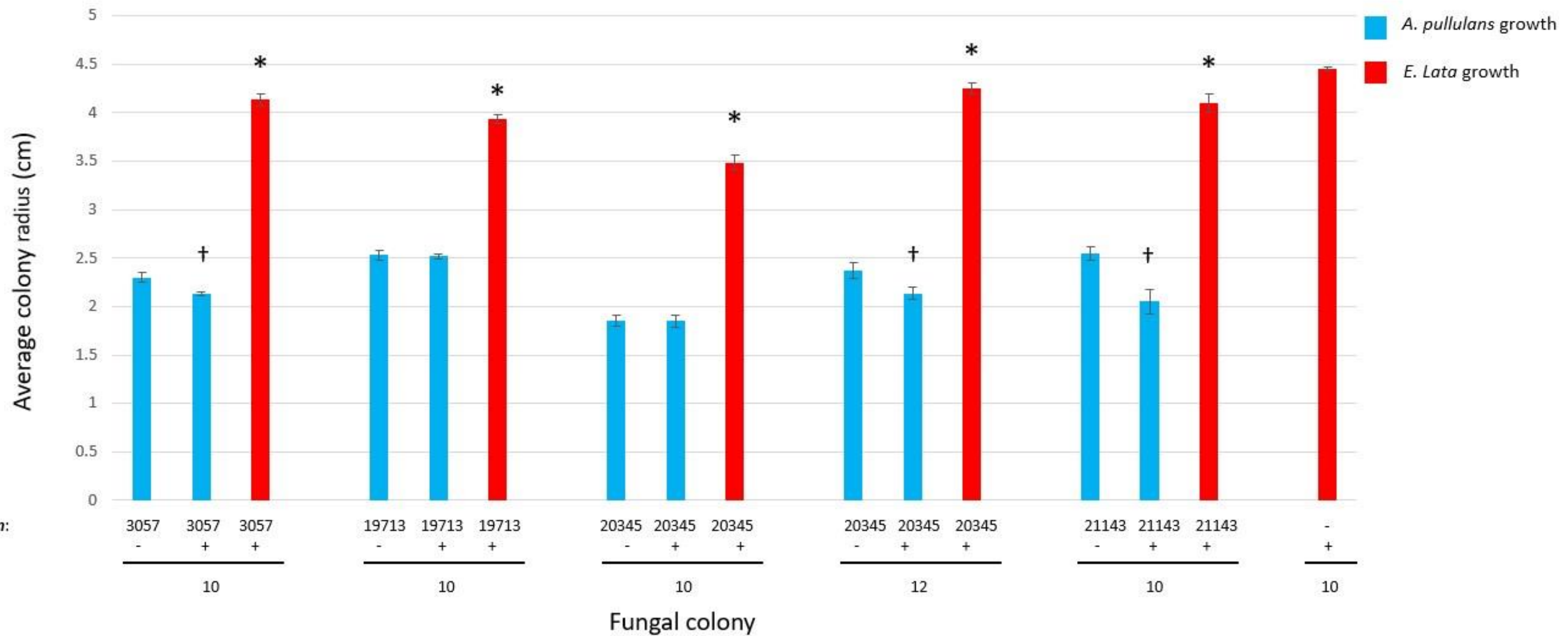


Figure 3.9. ***In vitro*** **analysis of** ***Aureobasidium pullulans*** **and** ***Eutypa lata*** **growth (n=6).** Either *A. pullulans* (one of four strains) or *E. lata* 20026 were cultured alone, or co-cultured on potato dextrose agar (PDA). The radii of colonies were measured when surface contact was first observed (10 dpi, or 10 dpi and 12 dpi for *A. pullulans* 20345). Standard errors were calculated for each column and ANOVA tests were performed at 95% confidence level. The * indicates that *E. lata* colony size was significantly reduced when co-inoculated with *A. pullulans* compared to *E. lata* inoculated alone; the † indicates that *A. pullulans* colony size was significantly reduced when co-inoculated with *E. lata* compared to *A. pullulans* inoculated alone.

3.3.2.2 *E. lata* alone and comparison with all four strains of *A. pullulans*

E. lata 20026 showed low variability in colony radius when cultured alone and was inhibited by all *A. pullulans* strains. At day 10, three of four *A. pullulans* strains except 20035 met colonies of *E. lata* 20026 on the co-culture plate. The average colony radius was 4.45 cm when *E. lata* was cultured alone, and it decreased to between 3.93 cm to 4.13 cm at day 10.

3.3.2.3 Comparison of *A. pullulans* and *E. lata* inoculated together compared to each alone

When dual-cultured with *E. lata*, some *A. pullulans* replicates started to show surface contact on plates from day 6. By day 6, colony sizes and shapes of *A. pullulans* and *E. lata* showed no difference in dual-cultures and single cultures. Hence all plates were cultured until surface contact occurred in all 6 replicates of treatment.

At day 10 dpi, three fungal strains *A. pullulans* 21143, 19713 and 3057 showed surface contact with co-inoculated *E. lata* colonies in all replicates. Three out of six replicates of *A. pullulans* 20345 has changed surface shapes of *E. lata* 20026 in dual-culture plates without surface contact, the other three replicates did not change or met *E. lata* colony (Figure 3.8). Colony radii of all replicates were recorded. Lengths of colony radius of *A. pullulans* 20345 and *E. lata* 20026 ranged from 0.2 cm to 0.4 cm when co-inoculated with *E. lata* (Figure 3.8). At day 10 dpi, the average colony sizes of *E. lata* were smaller in all co-inoculation treatments than *E. lata* cultured alone (Figure 3.9 and Appendix 1). *E. lata* was inhibited by the dual-cultured *A. pullulans* (all four strains) than cultured alone. *E. lata* showed the highest reduction size when dual-cultured with *A. pullulans* 19713 by 0.47 cm (inhibition percentage at 10.5%). Also, *A. pullulans* 19713 was the least inhibited *A. pullulans* isolate among all four strains.

At day 12 dpi, all replicates of *A. pullulans* 20345 met *E. lata* on dual-cultured plates. Colony radii of all replicates of *A. pullulans* 20345 dual-culture and by itself were measured Figure 3.8 and Appendix 1).

3.3.2.4 Statistical analysis for colony radii of *in vitro* culture tests

After measuring and recording colony radius, One-Way ANOVA was performed to determine the statistical significance. Comparison of inhibition effects of among *A. pullulans* strains and *E. lata* were made to understand what extent *A. pullulans* inhibits the growth of *E. lata in vitro*.

Two fundamental research questions were analysed by One-way ANOVA test for statistical significance at 95% confidence level (Table 3.5):

1. Does the inoculation of any of the four strains *A. pullulans* affect significantly *E. lata* colony size compared to inoculation of *E. lata* only?

2. Does the inoculation of *E. lata* affect significantly the colony size of any of the four strains of *A. pullulans* compared to inoculation of *A. pullulans* only?

The statistical analyses showed all four *A. pullulans* strains (include *A. pullulans* 20345 at both day 10 and day 12) co-inoculated with *E. lata* have a significant growth reducing the effect on *E. lata* colony radius, compared to medium inoculated with *E. lata* alone (Table 3.5). Similarly, in some cases *E. lata* also reduced *A. pullulans* colony size when both fungi were inoculated on the same medium. This latter phenomenon was dependent on *A. pullulans* strain: colony sizes of *A. pullulans* 3057, 20345 and 21143 were reduced by the co-inoculation of *E. lata*, there was no evidence of *A. pullulans* 19713 colony size reduction by co-inoculation of *E. lata* (Table 3.5).

Table 3.5. Statistical analysis of *Aureobasidium pullulans* and *Eutypa lata in vitro* colony sizes. Colony radii of *A. pullulans* and *E. lata* in single or dual inoculated PDA plates were measured when surface contact was observed at 10 dpi (for all fungi) and also at 12 dpi for *A. pullulans* 20345. Statistical significance was performed by one-way ANOVA test at a confidence level of 95%.

Fungal treatment		**Significant negative impact of *E. lata* on *A. pullulans* colony size: Yes/No (p-value)**	**Significant negative impact of *A. pullulans* on *E. lata* colony size: Yes/No (p-value)**
A. pullulans	*E. lata*		
3057	20026	Yes (0.0136)	Yes (0.00068)
19713	20026	No (0.7804)	Yes (<0.0001)
20345*1	20026	No (1)	Yes (<0.0001)
20345*2	20026	Yes (0.04366)	Yes (0.0079)
21143	20026	Yes (0.0068)	Yes (0.0065)

*1: No surface contact between colonies of the two fungal species was observed at 10 dpi.
*2: Surface contact between colonies of the two fungal species was observed at 12 dpi.

3.3.3 Culturing of *B. cinerea* and *E. lata in vitro*

Single and dual culturing of the non-BCA fungus *B. cinerea* with the phytopathogen *E. lata* was performed to demonstrate the unique BCA impact of *A. pullulans* and to exclude the potential effect of anti-fungal components secreted by fungus generally or whether competition for basic nutrients is a major mechanism to inhibit the growth of *E. lata*. As shown in Figure 3.1, this experiment cultured the non-BCA fungus and *E. lata* on the individual or the same plates in the same manner as the experiments performed for *A. pullulans* and *E. lata* (section 3.3.3). The non-BCA and *E. lata* experiment were performed to answer the question: Would non-BCA fungi also inhibit the growth of *E. lata*?

B. cinerea showed significantly faster growth rate than *E. lata* but with colony sizes that varied among replicates, ranging from 2.1 cm to 4.6 cm by day 4 (Table 3.6). Since *B. cinerea* occupied plates rapidly, the radii of *E. lata* on both dual-cultures and by itself were smaller than those in the antagonist tests with *A. pullulans*.

No inhibitory effect was observed before surface contact formed (data not shown). Pictures (Figure 3.10) and data (Table 3.6) showed that the radii of *B. cinerea* colonies are significantly larger than *E. lata*, in both co-inoculation treatments and control treatments.

The rapid growth of *B. cinerea* resulted in a large proportion of petri dishes colonised by this fungus and once the colonies of the two species met then both strains stopped growth. There was no evidence from this experiment for the inhibitory ability of *B. cinerea* on *E. lata* (Table 3.6). Despite a bigger range of each colony size for both fungi (Table 3.6) compared to that observed for the experiment with *E. lata* and *A. pullulans* trails (Appendix 1), the average radius of *E. lata* remained unchanged in single and dual inoculations (Table 3.6). Statistical analyses of these data showed no significant *in vitro* inhibitory effects of *B. cinerea* and *E. lata* against each other. No statistical significance support *B. cinerea* or *E. lata* has different radii when they were cultured together than cultured by themselves (Table 3.7). This supports our prediction: *A. pullulans* works differently from other fungi and has the potential to inhibit growth of *E. lata*.

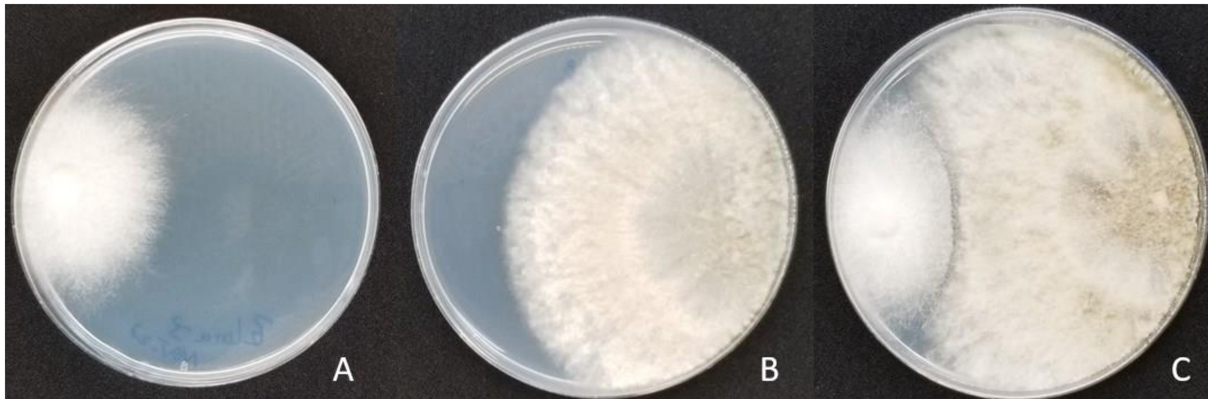


Figure 3.10. Colonies of *Eutypa lata* (A), *Botrytis cinerea* (B) and both (C) on PDA (n=6). Photos were taken when surface contact between *B. cinerea* and *E. lata* was observed when both fungi were inoculated on plate C (7 dpi). Either *E. lata* and *B. cinerea*, or *E. lata* with PDA medium, or *B. cinerea* with PDA medium were inoculated on 1 cm from the edge of culturing petri dish.

Table 3.6. *In vitro* colony radius of *Botrytis cinerea* and *Eutypa lata* in antagonistic tests (n=6). Data were recorded at 4 dpi. Statistical analysis was performed by one-way ANOVA at a confidence level of 95%.

Fungal treatment		Average colony size (cm)/standard deviation		Comments
B. cinerea	*E. lata* 20026	*B. cinerea*	*E. lata*	
+	+	4.2*/0.34	1.83*/0.30	Dual-culture did not significantly affect radii of *B. cinerea* or *E. lata*.
+	-	3.5*/0.99	-	
-	+	-	1.81*/0.23	

The * means *B. cinerea* has significantly larger colony radii than *E. lata* both in dual-culture (P<0.00001) and cultured by themselves (p=0.0023).

Table 3.7. Statistical analysis of *Botrytis cinerea* and *Eutypa lata in vitro* colony sizes. Colony radii of *B.cinerea* and *E. lata* in single or dual inoculated PDA plates were measured when surface contact was observed at 4 dpi. Statistical significance was performed by one-way ANOVA test at a confidence level of 95%

Fungal treatment		Significant negative impact of *E. lata* on *B. cinerea* colony size: Yes/No (p-value)	Significant negative impact of *B.cinerea* on *E. lata* colony size: Yes/No (p-value)
B. cinerea	*E. lata* 20026	No (0.133)	No (0.917)

3.4 Discussion

3.4.1 Strains of *A. pullulans* and *E. lata*

A. pullulans strain selection would preferentially have included those isolated from grapevines but we did not find such *A. pullulans* strains within the New Zealand ICMP. ICMP was the fundamental fungal storage organisation for us to search for specific strains in New Zealand. We did not check other sources: importing fungal species into New Zealand requires longer process and results in a higher cost than purchasing from ICMP. It can be helpful if we could apply *A. pullulans* strains initially found in grapevines: thus we are confident that these strains would colonize vines and they may behave better than other isolates.

Instead of grapevine derived isolates of *A. pullulans*, the *A. pullulans* strains chosen for the experiments in this research were from specimens that were first isolated from a range of plants or plant tissues (Table 3.1). *A. pullulans* 3057 (isolated from apple) was chosen because of a previous report of *A. pullulans* protecting apple trees from fungal diseases (Ippolito, El Ghaouth et al. 2000). *A. pullulans* 21143 was isolated from leaves of *Dodonaea viscoca* (Hopbush, or akeake for Maori name), a native plant of New Zealand that has tough and durable wood traditionally used for weapons by Māori. It was interesting to find that *A. pullulans* can be sourced from native plants of New Zealand and it brings the question of whether the species is native to New Zealand. *A. pullulans* 19713 was isolated from stem tissues of *Cytisus scoparius* (scotch broom), an exotic ornamental plant that became a weed in New Zealand for the lack of predator (Paynter, Buckley et al. 2016). Because *C. scoparius* distributes widely in New Zealand, it is possible that this *A. pullulans* strain also spreads widely nationwide. Similar to *A. pullulans* ICMP 19713, *A. pullulans* ICMP 20345 was isolated from stem tissues of another invasive plant *Ranunculus acris* (meadow buttercup). We looked up information of AureoGold (the commercial *A. pullulans* product for biocontrol) but did not find this specific strain at ICMP. We should have purchased some AureoGold product to test *A. pullulans* strains contained.

By contrast to *A. pullulans*, *E. lata* has been isolated from New Zealand grapevines. There were two *E. lata* strains purchased: ICMP 20026 and ICMP 20027. From a trail test of both strains on PDA medium, they showed similar growth rate, colony shape and structure (data

not shown). Thus, to reduce manual work, only *E. lata* 20026 was assessed in further experiments.

Strains of *A. pullulans* and *E. lata* had similar morphological features as described in other papers. The four strains of *A. pullulans* expressed different pigmentation development speeds and colour in this study after the first few days. It is common to have varied colony colours among *A. pullulans* strains (Zalar, Gostinčar et al. 2008). The four *A. pullulans* strains used in this study shared similar structures in colony shapes and hyphae status, while other studies claim that some strains may vary on hyphae and colony shapes (Zalar, Gostinčar et al. 2008).

The morphological observations of each fungal colony provided some evidence that the correct species had been received from ICMP however visual identification is not a definitive identification. Therefore, we confirmed the species using molecular methods (Section 3.3.1.2 and 3.3.1.3). All four strains of *A. pullulans* and the *E. lata* isolate were ultimately confirmed by either endpoint or real-time PCR. End-point PCR worked as an initial approach of the molecular experiment. It confirmed that primers AP19F and AP386R can be used to specifically amplify all four *A. pullulans* strains resulting in the expected product size. However end-point PCR failed to provide clear differentiation between positive and negative controls of *E. lata;* although the product was of the expected size. Real-time PCR (SYBR-Green) worked as a supplement to end-point PCR for *E. lata*. It differentiated positive and negative controls and the two different species (*E. lata* and *A. pullulans*) by measuring amplification curves and melt temperatures. Real-time PCR was faster and more sensitive than end-point PCR. One improvement of this identification process would be to sequence all PCR products and to compare those sequences with deposited in genome databases such as National Center for Biotechnology Information (NCBI) and related publications.

3.4.2 Inhibition effects of *A. pullulans* on *E. lata*

All four *A. pullulans* strains showed significant inhibitory effects on *E. lata* colony sizes *in vitro* although the inhibitory rates varied depending on which *A. pullulans* strain was used. Each *A. pullulans* strains reduced *E. lata* colony sizes from 7.2% to 11.7%. Thus, the major result from this is that in all tests: *A. pullulans* can efficiently inhibit the growth of *E. lata in*

vitro. At a strain level, *A. pullulans* 19713 expressed the best inhibitory efficacy among the four strains and *A. pullulans* 20345 strain had the least efficacy in day 10. Efficacy of *A. pullulans* 20345 started to occur in day 12 (Figure 3.9 and Appendix 1).

A. pullulans 20345 is the slowest growing isolate of all four strains. By day 10, there was no surface contact between co-inoculated colonies. Noticeably, *E. lata* also reduced growth speed when dual-cultured with *A. pullulans* 20345. This increased probability that *A. pullulans* 20345 can inhibit the growth of *E. lata* in addition to directly competing for nutrients and niches (maybe by secreting antibiotic compounds). Other *A. pullulans* strains did not show this effect but it was more possible that other *A. pullulans* strains grow fast and their colony growth has covered the effect of secreting antibiotic compounds. In order to further clarify the antagonistic properties of *A. pullulans* 20345 its culturing time was prolonged to day 12.

Apart from the direct comparison of colony sizes when surface contact occurred, the fungi also changed their colony shapes upon contact with the other species. *A. pullulans* 20345 had the most obvious impact on the other species as it appeared to alter the *E. lata* colony shape prior to direct contact (Figure 3.3). This altered phenotype of *E. lata* prior to contact with *A. pullulans* 20345 may indicate the presence of a secreted chemical compounds that interferes with the growth of *E. lata*. *A. pullulans* has shown the ability to produce a wide range of organic compounds against fungal pathogens, such as alcohols, pullulan, amylases, cellulase, etc. (Di Francesco, Ugolini et al. 2015). Many of these secreted compounds are from the group of alcohols that have antibiotic effects.

Non-BCA dual-culture emphasised the unique effect of *A. pullulans* on *E. lata*. Dual-culture of *B. cinerea* did not reduce colony size of *E. lata*, indicating some chemical inhibitory effect present instead of simply competing for nutrients and living space.

Some studies have demonstrated *A. pullulans* can produce extracellular polymeric substances (EPS), sometimes known as biofilm, as a biocontrol against post-harvest diseases (Ravella, Quiñones et al. 2010). In citrus fruits, *A. pullulans* expressed killer activity *in vitro* against *Geotrichum citri-aurantii* (causal agent of citrus sour rot), indicating the death of pathogenic fungi cells (Ferraz, da Cunha et al. 2016). In addition, Ferraz et al (2016) also

identified chitinases produced by *A. pullulans*, which could degrade pathogen cell wall and use its components as carbon resource.

3.4.3 *A. pullulans* inhibitory mechanisms against *E. lata in vitro*

Any mechanisms for how endophytes inhibit the growth of pathogens identified in sections 1.4.4 to 1.4.6 above are possible reasons in this test, except for triggering plant defense system and degradation of virulence factors or phytotoxins (Table 1.2). Moreover, because of the limitations of time in this research chemical analysis was not undertaken the discussion is mainly based on colony size, shape and growth rates of both fungal species.

Another study showed that *A. pullulans* cultural filtrates significantly reduced *Monilinia laxa* (a common fungal pathogen of peaches) conidia germination rate and decreased fungal germ tube *in vitro* tests (Di Francesco, Roberti et al. 2015). Though my project did not focus on conidia germination or germ tube growth, Di Francesco's study demonstrates the synthesis of active antimicrobial compounds by *A. pullulans* plays a role in competing (or inhibiting) fungal pathogens.

Competition for basic growing space (niche competition) and nutrients (resource competition) between plant microbes are both methods for endophytic fungi BCA activity (Elmer and Reglinski 2006). Competition is not the only mechanism occurring in the *in vitro* experiments described in this chapter. *A. pullulans* strain 3057, 21143 and 20345 share similar inhibitory rates on *E. lata*, despite the growth of strain 3057 being much slower than the other two strains (section 3.2.4). However, *A. pullulans* 19713 showed a similar growth rate as 3057 and 21143, but it has the strongest inhibitory effect against *E. lata* without being affected by the pathogen. This may suggest that *A. pullulans* 19713 has a more predominant status in competition in comparison with other strains.

This experiment could be improved by adjusting culturing conditions. Many fungi express different enzyme activities under stress, such as lack of nutrients, higher environmental temperature or existence of specific antibiotics (Klein and Kupper 2018). Such environmental variations could have positive or negative effects on *A. pullulans* and *E. lata*. According to Klein and Kupper (2018), *A. pullulans* expresses enhanced antagonistic action

and even lower mycelial lengths when the concentration of some micronutrients (ammonium sulfate, boric acid and copper sulfate being the three most influential effectors) are artificially increased (Klein and Kupper 2018).

3.4.4 *E. lata* also inhibited *A. pullulans* colony sizes

E. lata reduced *A. pullulans* colony sizes. *E. lata* has significantly reduced colony radii for three out of four *A. pullulans* strains: only *A. pullulans* 19713 stayed unaltered. This is an interesting aspect to consider, that there might be some differences in spectres among strains. One possible explanation for this phenomenon is the competition for basic nutrient and space between two fungi although one would not anticipate such a high degree of variation between effects of different *A. pullulans* strains if this were the case.

Botrytis cinerea was used as a non-BCA in the experiments within the current research. *B. cinerea* is a well-known fungal pathogen that produces a range of cell wall degrading enzymes and toxins (Williamson, Tudzynski et al. 2007) thus it is harmful to a wide range of crops (Williamson, Tudzynski et al. 2007, Plesken, Weber et al. 2015). However, *B. cinerea* has no protective effect reported to date (AbuQamar, Moustafa et al. 2017). Likewise, *B. cinerea* is assumed to have no anti-fungal effects against *E. lata*. In comparison to the antagonistic tests conducted with *A. pullulans* and *E. lata*, the non-inhibitory result of *B. cinerea* on *E. lata* revealed no BCA activity, as anticipated. The use of *B. cinerea* in the dual cultures with *E. lata* compared with *A. pullulans* provides some evidence against any competition for basic nutrient and space between two fungi. *B. cinerea* grew faster than *A. pullulans* yet *B. cinerea* did not inhibit *E. lata* growth.

A possible explanation for *B. cinerea* not affecting *E. lata* is a mutualism among fungal phytopathogens. Most fungal phytopathogens share similar pathways to obtain nutrients from plants. They can either simply secrete enzymes to degrade plant cell walls (Paccanaro, Sella et al. 2017) or suppress plant defense responses (Masachis, Segorbe et al. 2016). Both ways have the potential to favour the growth of other fungal pathogens in the same plant. This ‘co-operation’ among phytopathogens may explain why GTDs often occur as a multiple infection of several fungi. However, this theory does not explain the interaction observed here, *in vitro.*

B. cinerea was different from other fungi cultured in this chapter. Their colony sizes and growing speed varied among replicates, significantly (Table 3.6). But the overall trend was that *B. cinerea* grows faster when two species were inoculated onto the same culture. It is possible that *E. lata* and *B. cinerea* form a symbiotic relationship and therefore benefit the growth of one or the other.

It was hard to compare the growth rate of *E. lata* with *B. cinerea* and *A. pullulans* because surface contact occurred after different days. In day 4, *E. lata* and *B. cinerea* colonies have covered entire plate, but we did not record colony radii in dual-culture test for *E. lata* and *A. pullulans*.

3.4.5 Difficulties encountered in this study

Although the method is conceptually simple, it was challenging to measure the colony radii on the petri dishes. This was particularly the case for *E. lata*. *E. lata* has radial mycelium at grow from its inoculation point to all direction on plates. The density of the mycelium structure decreases at points more distal from the inoculation point. Therefore it was hard to discriminate whether a very thin fungal mycelium was present or should be recorded. Thus, the definition of 'colony' was not always clear. Within this research, the colony radius was standardised as from inoculation point to the edge of clear, high density colony of the straight line to the opposite inoculation point. Different measuring methods would result in a distinct measurement that may not be comparable with the results described in this chapter.

3.4.6 Future directions

This chapter has several aspects that could be improved or extended in future research. The growth rates varied among fungal strains and treatments and the current study only focused on the final results of colony radii and shape. To gain a better understanding of the growth of each colony growth status could be recorded day by day to produce a growth curve. With such a curve we could obtain finer details of the changes of fungal growth in the single and dual inoculations. This information might assist in better understanding the fungal

interactions including whether inhibitory effects existed well before the two species physically interacted. Thus the timing and location might be determined to sample the PDA for growth inhibitory compounds.

The *A. pullulans* strains selection and tested in this chapter did not include any isolates from grapevines. Therefore, the experiments described do not mimic what might be occurring in grapevine trunks or represent future *A. pullulans* application. As described previously (section 1.4.5), *A. pullulans* is the major component of non-commercial and commercial BCAs that can be used to prevent a number of fungal phytopathogens (Ippolito, El Ghaouth et al. 2000, Pinto, Custodio et al. 2018). One of the commercial products named 'Aureo Gold' was based on live isolates of *A. pullulans* but different strains (KVH 2018). Although *A. pullulans* strains in our research varied in original species and morphological features, it would be more meaningful to perform the research described above using the same strains as in Aureo Gold (*A. pullulans* YBCA5).

This research contained only one strain for *E. lata* in both *in vitro* and *in vivo* tests. This was a reasonable mitigate the limited study time and funding. We prioritised the research on *E. lata* 20026 which was isolated from grapevine roots (Table 3.1). To identify if all *E. lata* strains affecting New Zealand vineyards share similar antagonism by *A. pullulans in vitro*, testing other strains collected from other regions nationwide and different tissues of grapevines would enhance our overall knowledge.

Knowing what effects of glyphosate on the microbiome, *A. pullulans* and *E. lata* is another future approach for this study. Since most vineyards apply glyphosate to control ground cover, the impact of this and other herbicides on both fungi species would increase our understanding of the vineyard ecosystem. As discussed in Chapter 2.4.4, glyphosate can affect the abundance of fungal endophytes and pathogens. A future approach for in vitro research is to add a range of concentrations of glyphosate into the PDA medium to determine the direct impacts on the fungi of interest and to simulate the presence of herbicide in vineyards.

A combination of both inhibitions on colony sizes and alternation on *E. lata* shapes suggested further research of the interaction of *A. pullulans* and *E. lata* was worthwhile. The research described here did not reveal the mechanisms of inhibition observed in the *in vitro*

tests. *In vitro* test alone are insufficient to uncover more information about how these fungi interact or what would happen in real plants. Thus further studies *in planta* are required to investigate protective effects of *A. pullulans* as a potential BCA for use in grapevine production.

4. *In planta* antagonistic tests of *A. pullulans* against *E. lata*

4.1 Introduction

Beyond the scope of *in vitro* tests, *in planta* protection tests were performed to uncover more information about the protective potential of *A. pullulans* against *E. lata* in the plant ecosystem (Figure 4.1). Canes collected from young Sauvignon blanc grapevines played important roles in this chapter. They were treated with fungal plugs and grow in shade house, to study the presence of leaf and root, stain size and fungal presence of *A. pullulans* and *E. lata.* Hence a combination of *in silico*, *in vitro* and *in planta* results would reveal the potential of *A. pullulans* as BCA against *E. lata* and help to design new biocontrol methods to GTDs.

The research described in this chapter involving grapevine cane inoculation and maintenance in shade house conditions was undertaken at PFR Blenheim site, under the supervision of Dion Mundy and Rebecca Woolley (Plant and Food Research). The research involving DNA isolation and analysis was performed at PFR Auckland site under the supervision of Robin MacDiarmid and Karmun Chooi. Due to the difficulty of removing fungal cultures from the PC2 laboratory, PFR Auckland the same four strains of *A. pullulans* and *E. lata* (Table 4.1) were re-ordered and delivered to PFR, Blenheim directly.

This chapter addressed Research aim 3: Identify the strain of *A. pullulans* that provides the best biocontrol of *E. lata* and GTD symptoms within *in planta* conditions (section 1.5).

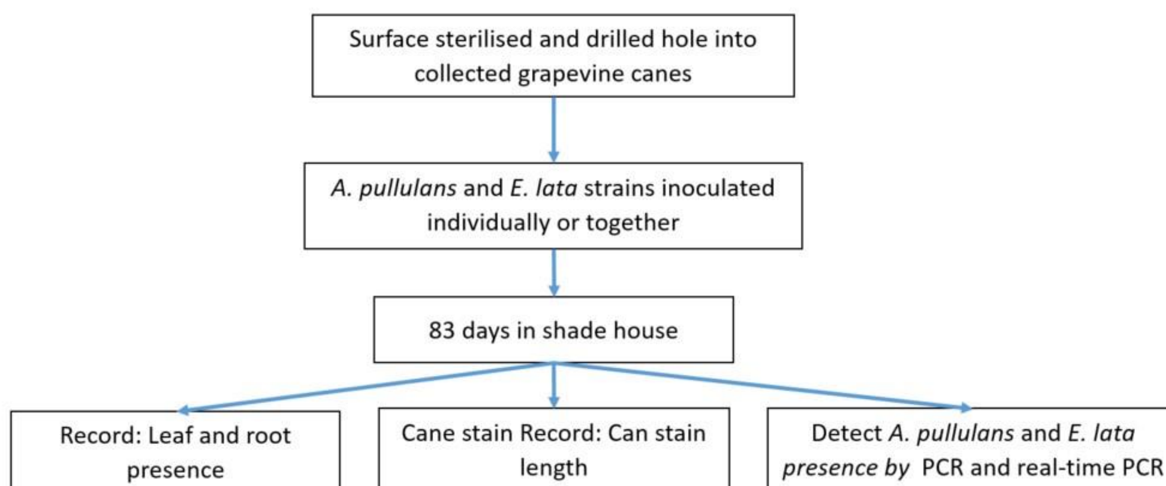


Figure 4.1. Flow chart of experiment steps of *in planta* test in Chapter 4. *In planta* experiments were performed at PFR Blenheim and PFR Auckland.

4.2 Materials and methods

4.2.1 Grapevine cane models establishment

Grapevine cane samples were cut from cane collections, surface sterilised and then drilled one hole prior to inoculation (Figure 4.2). Canes with two nodes were cut from one-year-old Sauvignon blanc grapevines from PFR Marlborough sites. Canes could not cut into uniform length because they were collected randomly from the vineyard and comprised nodes at different spacing. Each cane was selected such that the length between the two nodes ranged from 7 cm to 15 cm. Canes were cut such that at least 2 cm of cane tissue below the lower node and 2 cm above the higher node was reserved in each cane. Then these canes were soaked in 1% NaClO solution for 15 min to surface sterilise their external surfaces. Following sterilisation, the canes were soaked in sterile water overnight.

Figure 4.2. Inoculation of fungal strains into grapevine canes. A. Canes were inoculated with 5 mm drill bits; B. Each cane contained one hold for around halfway through cane for fungal inoculation; C. Inoculated canes were sealed with parafilm.

4.2.2 Inoculation of *E. lata* and *A. pullulans* strains into model cane systems

Surface sterilised cane samples were drilled in the middle of the inter-bud area using a 5 mm diameter drill bit on an electric drill. One hole was drilled into every cane to a depth of half the cane width (Figure 4.2B). Each hole was used to place the inoculation plugs. Mycelial plugs of *A. pullulans* (four strains) and/or *E. lata* 20026 were inoculated into the holes according to the treatments outlined in Table 4.1. *A. pullulans* was always the first fungus to be inoculated when both fungi were inoculated into a single cane. Between inoculations, equipment was surface sterilized by 80% ethanol and wiped with a paper towel to minimise contamination. Finally, the inoculation point of the canes was sealed with parafilm and the cane was placed into pots (see section 4.2.3).

The inoculum was cut from a 7-day-old culture of the required fungal strain growing rapidly on PDA medium. Each experimental treatment contained 12 to 14 replicates. For each treatment, all inoculated canes were recorded for leaf and root presence and cane stain size. However, only 10 replicates of each treatment (randomly selected) were used for DNA isolation and subsequent fungal detection. The canes that were visually observed but not molecularly studied were marked as "testers" and used to optimise DNA extraction methods.

Table 4.1. Fungal strains inoculated in each treatment group in grapevine cane models. Each treatment contained one of the following plugs: One *A. pullulans* strain and one *E. lata* 20026 (treatment groups 1-4); Two PDA medium (treatment group 5); One *A. pullulans* strain and PDA (treatment groups 6-9) or One *E. lata* 20026 with one PDA medium (treatment group 10).

Treatment group	*A. pullulans* strain	*E. lata* strain
1	3057	20026
2	21143	20026
3	19713	20026
4	20345	20026
5	/	/
6	3057	/
7	21143	/
8	19713	/
9	20345	/
10	/	20026

4.2.3 Inoculated cane samples growth and collection

Inoculated canes were placed into growing pots (16 cm high, 5 cm x 5cm; 400 ml in total) filled with a mixture of perlite and vermiculite (1:1 (v/v)) and placed in a plastic tray within a shade house belonging to Nelson-Marlborough Institute of Technology, Blenheim. All replicates were placed in the same shade house but distributed across six plastic trays as depicted in Figure 4.3. Samples were placed closely (adjacent pots were touching each other) and growing conditions were controlled to maintain all pots under the same environmental condition. Samples inoculated on 4th December 2018 and were maintained under the shade house conditions until 25th February 2019 for a total of 83 days.

7	7	8	8	8
7	7	8	8	8
7	7	7	8	8
7	7	7	8	8

3	3	4	4	4
3	3	3	4	4
3	3	3	4	4
3	3	4	4	4

3	4	8	5
5	2	9	2
1	5	3	7
N/A	10	6	8
N/A	1	5	5
6	9	5	6

1	1	5	5
5	5	6	6
5	6	6	6
6	5	5	5
6	6	5	6

1	1	2	2	3
1	1	2	2	2
1	1	2	2	2
1	1	2	2	2

10	9	10	10	9
10	10	10	9	9
10	10	9	9	9
10	10	9	9	9

Figure 4.3. Fungal treatments of inoculated canes and the location of each cane within the shade house. Treated canes were positioned in growing pot on plastic trays to distribute the treatments across the growing area. Each cell depicted represents one cane and the number represents the fungal treatment described in Table 4.1.

4.2.4 Foliar and root presence of treated canes

After 83 days of maintenance in the shade house, the canes were collected and washed to remove surface soil. The canes were then cut with pinchers horizontally at each inoculation point to divide each cane into upper and lower parts; the equipment was surface sterilized in 80% ethanol to avoid contamination between canes.

The presence of leaf and roots growing from the canes was recorded along with the length of the upper and lower stain size. Leaf presence was visually assessed and recorded as

“present” (marked as “1”) or “absent” (marked as “0”) regardless of the number of leaves or foliar symptoms. Root existence was measured and recorded the same way as leaf presence. Results of vines with leaf or roots were analysed by One-Way ANOVA Calculator (Tang 2019) to study the statistical impact of fungal inoculation on leaf of root presence. Tests were performed at 95% confidence level.

4.2.5 Measurement and recording of stain length

To determine the stain size each cane was cut longitudinally and canker or stain which appeared close to inoculation wound was observed and measured. The distance between the proximal edge of canker and the inoculation point was measured and recorded for every cane in each group (Figure 4.4). Grapevine canes inoculated with fungal strains were compared with negative control treatments to determine the impact of PDA or fungal inoculation on cane stain or cankers. If a cane was completely discoloured from inoculation point to the distal or proximal bud, the stain size was recorded as 4 cm for its upper or lower stain recording, as appropriate, so as to minimize the influence of each individual cane length.

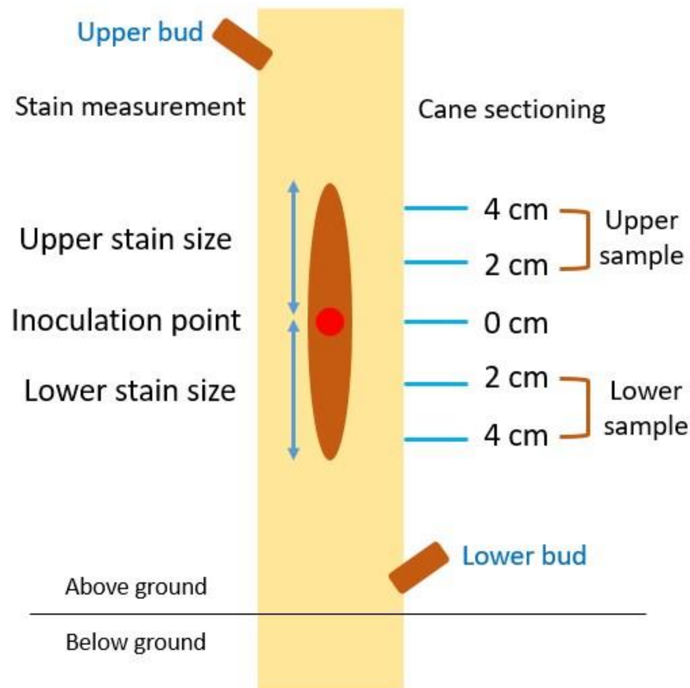


Figure 4.4. Indication of grapevine cane used for fungal inoculation test. Stain size was measured vertically for both above and below inoculation point. Cane samples were collected at 2 cm to 4 cm away from inoculation point for DNA isolation (detailed in section 4.2.6).

4.2.6 Molecular analysis of *in planta* fungal presence status in treated canes

4.2.6.1 Grapevine cane homogenisation and DNA extraction

Cane samples were then cut at 2 cm to 4 cm both above and below inoculation point for DNA extraction Because most existing homogenisation methods were designed for leaf samples or cane samples in a near powder condition, we had to optimise the homogenisation process to maximise the time and subsequent DNA isolation efficacies. Each cane sample was placed in a mortar and made brittle by chilling to -195˚C with liquid nitrogen then smashed gently with the pestle and ground rapidly until it became a fine powder.

After grinding, the powder was transferred into 2 ml strength enhanced tubes (Invitrogen, USA). Then multiple 2 mm metal beads were added and the powder was further

homogenisation with the Omni Bead Ruptor 24 (Omni International Inc, Tulsa, OK, USA) that was set at a speed of 3.7 m/s for 15 seconds, add through 10 cycles.

4.2.6.2 Total grapevine cane DNA extraction and quality measurement

After homogenisation, total DNA was extracted with the CTAB methods described in section 3.2.2.2. Following total DNA isolation, 1 µL DNA extracted from each grapevine cane sample was measured by DS-11 Fluorometer using the "dsDNA" software (DeNovix, USA). DNA concentration (ng/µL) and quality (value of A260, 260/230 and 260/280) were recorded for each sample.

4.2.6.3 End-point PCR tests for sensitivity test of *A. pullulans* and *E. lata* in grapevine DNA

To compare the sensitivity and reliability of PCR results both end-point PCR and real-time PCR were assessed. Before amplification, all grapevine DNA were standardised to 20 ng/µL for use as DNA templates. If the DNA concentration was lower than 20 ng/µL then they were not considered for the following analyses.

End-point PCR for *A. pullulans* and *E. lata* followed the same protocols as described in section 3.3.1.2 with minor modifications of reagents. For use in this section we added Bovine Serum Albumin (BSA, Sigma, Germany), final concentration 0.2 mM, into the master-mix of *A. pullulans* PCR so as to reduce the impact of plant-derived inhibitors. The master-mix used for *E. lata* identification by end-point PCR contained no BSA but instead included 6.60 µL of water for each reaction (Table 4.2), while the other components were the same as master-mix used for *A. pullulans*.

Table 4.2. Contents for end-point PCR of *Aureobasidium pullulans* and *Eutypa lata* from grapevine DNA. Volumes listed are for a total reaction volume of 10 µL**.** The * indicates different water amount per reaction for amplification of *A. pullulans* and *E. lata*; the † indicates BSA added only for amplification of *A. pullulans*.

Reagent	Concentration	Volume per reaction (µL)
Water*		6.40* (*A. pullulans*)
		6.60* (*E. lata*)
10 x DNA Buffer	10	1.00
dNTPs	10 mM	0.30
Magnesium	50 mM	0.50
Forward primer	20 mM	0.20
Reverse primer	20 mM	0.20
BSA†	10 mM	0.20†
Taq DNA polymerase	5 U/µL	0.20
Template	20 ng/µL	1

Protocols for grapevine PCR and Gel electrophoresis were the same as section 3.2.3.2.

4.2.6.4 Sensitivity tests of *A. pullulans* and *E. lata* by endpoint PCR

For the sensitivity tests for end-point PCR, each reaction contained 1 µL of pure DNA template of *A. pullulans* 3057 or *E. lata* 20026. The amount of DNA decreased for both fungi by performing a serial dilution to achieve 20 ng/µL, 2 ng/µL, 0.2 ng/µL and 0.02 ng/µL. PCR protocols were the same as section 4.2.6.3 for each fungus.

4.2.6.5 Sensitivity tests of *A. pullulans* and *E. lata* by real-time PCR

Sensitivity tests were also performed by real-time PCR for identification of both *A. pullulans* and *E. lata*. Two different methods and related materials were used to identify *A. pullulans* or *E. lata*. Amplification of *A. pullulans* used the TaqMan Probe real-time method with Taqman probe: APFTam1: 5’-ACCCCAACTTCGGAAGGGT-3’. *E. lata* used SYBR Green method that required no probe, as described in section 3.2.3.4.

The *A. pullulans* real-time PCR protocol that was applied was as follows: Preliminary denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing-extension at 60°C for 30 s, with an additional fluorescence acquisition step at the end of each extension (Chan, Puad et al. 2011). Each 10 µL reaction contained 3.55 µL water, 5.00 µL 5x PerfeCTa qPCR ToughMix (Quantabio, USA), 0.15 µL of AP19F (forward primer), 0.15 µL AP386R (reverse primer) and 0.15 µL Taqman probe APFTam1: 5'-ACCCCAACTTCGGAAGGGT-3'.

4.2.6.6 Real-time PCR for identification of two fungi from extracted grapevine DNA

All grapevine samples were tested for the presence of detectable *A. pullulans* and *E. lata* by real-time PCR. The same protocols as described in section 4.2.6.8 were applied for this purpose. The reaction for each sample was performed in two replicates, with 6 wells of Super-pure water (Invitrogen, USA) as negative controls.

Cq values of different templates were compared with Cq value of negative control, calculated as ΔCq. Real-time PCR for *A. pullulans* took ΔCq>5 as positive, while amplification for *E. lata* considered both ΔCq and melting temperatures.

4.2.6.7 One-way ANOVA test

Statistical analysis for Chapter 4 applied the One-way ANOVA test. The calculator was provided by Social Science Statistics (https://www.socscistatistics.com/tests/anova/default2.aspx).

4.3 Results

4.3.1 *In planta* inoculation results in grapevine canes

Results of *in planta* protection effects were measured by three aspects: Leaf and or root presence, cane stain size, and fungal movement as identified by both end-point PCR and real-time PCR.

4.3.1.1 Visual observation for leaf and root presence

All inoculated canes (n=122) including ‘tester’ canes which were not to be assessed by molecular methods were visually assessed for leaf and/or root presence. Since numbers of roots or leaves were difficult to determine quantitatively due to different sizes of each their presence were instead recorded qualitatively as either present or absent (Table 4.3).

In Figure 4.5, the respective cells (each representing a cane in a pot) that contained a cane that bore leaves were marked in green. Only 22 out of 122 canes bore one leaf or more. Of those leaf-bearing canes, 17 of 22 (77.2%) were inoculated with both *A. pullulans* (either one of the four strains) and *E. lata*. The tray on the bottom left corner contained 13 of these 22 (59%) leaf-bearing canes.

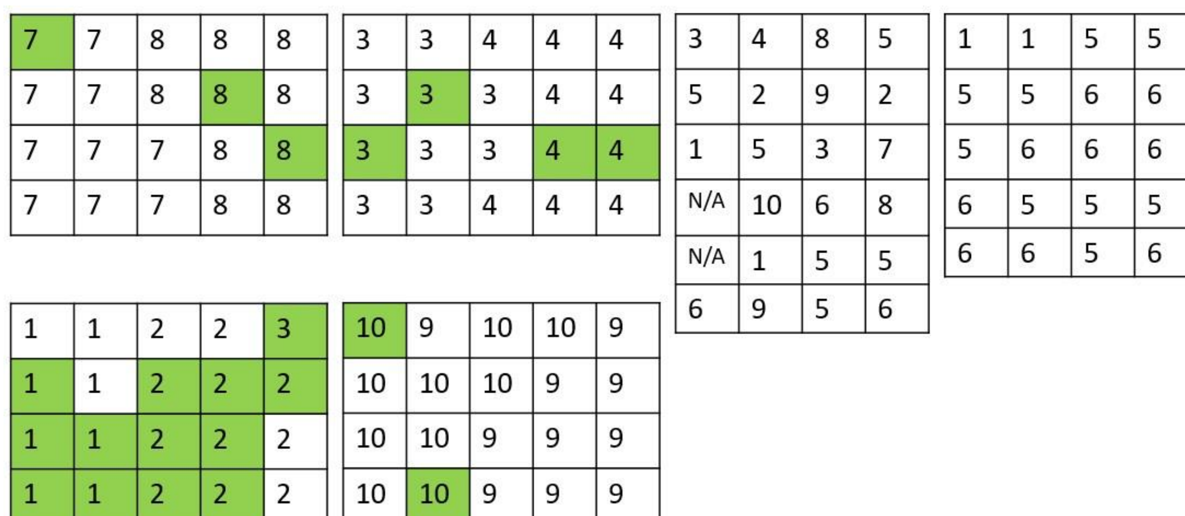


Figure 4.5. Location of treated canes that grew within a shade house with coloration of those bearing one or more leaf at 83 days post inoculation (83 dpi). Each treated cane was grown in a growing pot (16 cm high, 5 cm x 5cm) and placed in plastic trays. Colour of cell represents cane status: green means at least one leaf was identified; white means no leaf

was found. The number on each cell represents fungal inoculation treatments as defined in Table 4.1.

Root presence showed similar results to the results for the presence of at least one leaf (Table 4.3): canes inoculated with *A. pullulans* 3057/21143 and with *E. lata* had the highest rate root presence. Except for one cane in treatment 6 (*A. pullulans* 3057 only), all other canes with at least one root present also bore at least one leaf. One of the canes in treatment 6 (*A. pullulans* 3057 only) had at least one root but no leaves.

Table 4.3. Numbers of treated canes containing at least one leaf or root (n=122, 83dpi). Numbers represent canes with leaves or roots/total treated canes.

Treatment group	Fungal Treatment		Number of canes with leaves	Number of canes with roots
	A. pullulans	*E. lata*		
1	**3057**	20026	5/12	5/12
2	**21143**	20026	7/13	6/13
3	**19713**	20026	3/13	3/13
4	**20345**	20026	2/12	1/12
5	**/**	/	0/12	0/12
6	***A. pullulans* 3057**	/	0/12	1*/12
7	***A. pullulans* 21143**	/	1/12	0/12
8	***A. pullulans* 19713**	/	2/12	0/12
9	***A. pullulans* 20345**	/	0/12	0/12
10	**/**	20026	2/12	1/12

The* represents this cane was identified with roots but did not have any leaves.

4.3.1.2 Cane stain length

Stain length was measured from the inoculation point to the longest edge of each stain both above and below the inoculation point and recorded separately.

Cane stain was observed in all groups but the rate varied among treatments. A considerable number of canes (n=7 out of 12) showed stains when inoculated with only the two PDA medium treatment (Table 4.4). Canes inoculated with *A. pullulans* 21143 alone had the lowest stain rate at 8.3%. A total of 83% of canes inoculated with *A. pullulans* 20345 and *E. lata* expressed some extent of staining and this treatment gave the longest average stain length at 4.4 cm (Figure 4.6).

Table 4.4. Average stain size of different treatments in inoculation tests. Each treatment contains 12~13 replicates (n=122, 83 dpi).

Treatment group	**Fungal treatments**		**Stained canes /total canes**	**Average stain length (cm)**	**Standard deviation**
	A. pullulans	*E. lata*			
1	3057	20026	5/12	1.55	2.36
2	21143	20026	5/13	1.93	2.72
3	19713	20026	6/13	2.33	2.80
4	20345	20026	10/12	4.40	3.07
5	/	/	7/12	3.33	3.64
6	3057	/	6/12	2.58	3.26
7	21143	/	1/12	0.17	0.58
8	19713	/	7/12	2.21	2.94
9	20345	/	5/12	1.73	2.61
10	/	20026	6/12	2.13	2.70

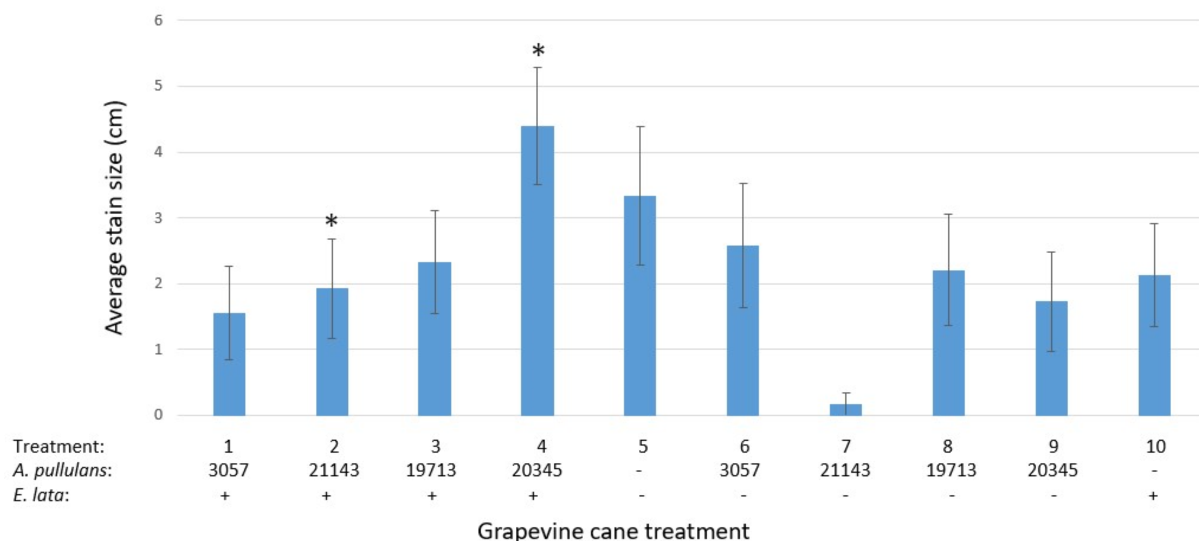


Figure 4.6. Average stain sizes within grapevine trunks inoculated with either *Aureobasidium pullulans* and/or *Eutypa lata* 20026, or neither (83 dpi). Potted grapevine budwood sticks were inoculated with pure fungi cultured from Potato dextrose agar (PDA, 21 dpi). Each budwood stick contained either one *A. pullulans* plug and one *E. lata* plug (lane 1-4), or one *A. pullulans* plug and one PDA medium plug (lane 6-9), one *E. lata* plug and one PDA medium plug (lane 10) or two PDA medium plugs (lane 5). Standard errors are shown on columns from ANOVA tests performed at 95% confidence level. The * indicates that *A. pullulans* significantly increased average stain size when inoculated with *E. lata*, compared to samples inoculated with *A. pullulans* alone.

4.3.2 End-point PCR of *A. pullulans* and *E. lata*

4.3.2.1 Inhibitory effect of grapevine DNA on *A. pullulans* identification by end-point PCR

A trial experiment of different proportion of *A. pullulans* diluted in water or grapevine samples were carried out to clarify if grapevine DNA could inhibit amplification of *A. pullulans*. An interesting result occurred that when diluted with water, *A. pullulans* can be detected at the lowest concentration at 5 ng/µL, but this minimum concentration increased to 10 ng/µL when diluted with grapevine DNA (Figure 4.7).

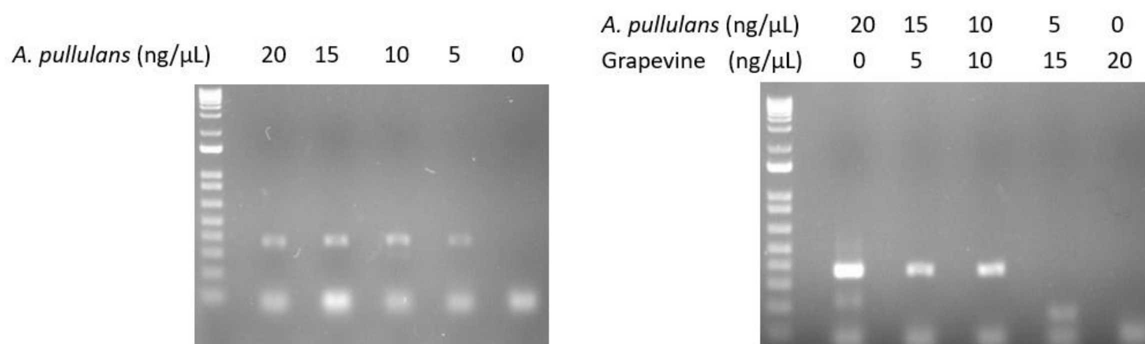


Figure 4.7. PCR results of *Aureobasidium pullulans* 3057 DNA diluted in water and grapevine extraction. In the left figure *A. pullulans* DNA concentration ranges from 0 ng/µL to 20 ng/µL diluted with water. A minimum of 5 ng/µL was sufficient for PCR identification. In the right figure *A. pullulans* DNA was diluted with grapevine extraction, a total DNA concentration was managed at 20 ng/µL in each tube. Minimum *A. pullulans* concentration was at 10 ng/µL (with grapevine DNA also at 10 ng/µL).

4.3.2.2 Sensitivity of *A. pullulans* identification with end-point PCR

The application of end-point PCR for the detection of *A. pullulans* showed consistent sensitivity through a serial dilution when *A. pullulans* DNA (sourced from a pure culture) was diluted either in water or grapevine DNA (10 ng/µL, Figure 4.8A and B). Brighter bands were observed when *A. pullulans* DNA at 20 ng and 2 ng was diluted in water (Figure 4.8A) rather than in grapevine DNA (Figure 4.8B). In Figure 4.8, clear bands are evident when *A. pullulans* DNA was at 0.2 ng, either when the target *A. pullulans* DNA was diluted in water or 10 ng/µL grapevine DNA (Figure 4.8A and B). These sensitivity results were consistent in all replicates,

thus the endpoint PCR sensitivity for *A. pullulans* under the conditions used in this assay is 0.2 ng target DNA.

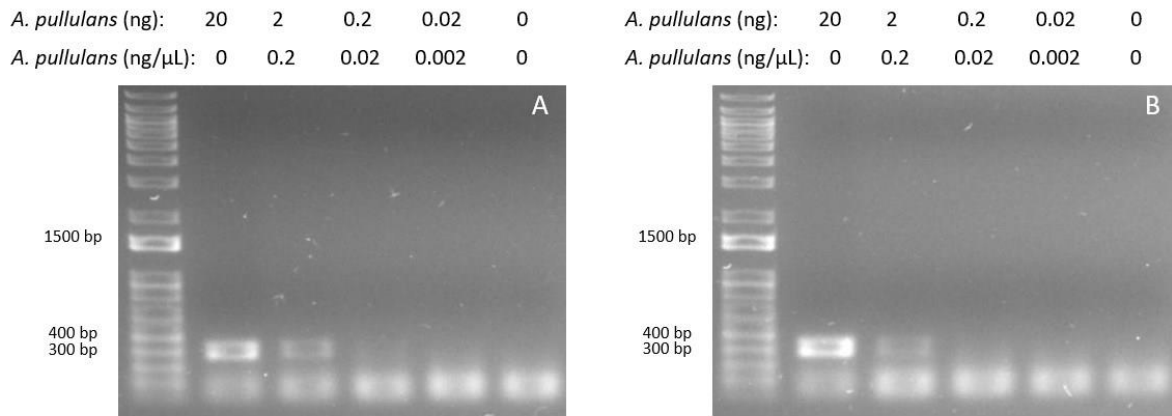


Figure 4.8. The sensitivity of endpoint PCR of *Aureobasidium pullulans* in reducing DNA amount diluted in water or grapevine DNA. Each reaction contains 1 µL template of *A. pullulans* 3057 pure DNA at an amount of 20 ng or 2 ng or 0.2 ng or 0.02 ng, with one water control. *A. pullulans* DNA was diluted in either water (A) or 10 ng/µL grapevine DNA (B).

4.3.2.3 Sensitivity and cross-contamination of *E. lata* as detected by endpoint PCR

The sensitivity test described for *A. pullulans* (section 4.3.2.1) was repeated for *E. lata* but bands of the expected size randomly appeared in water controls as occurred earlier (section 3.3.1.2). Many aspects were altered during an extensive period of troubleshooting however, the same result kept occurring even all reagents were changed (Figure 4.9). Thus, it was decided that endpoint PCR methods were not reliable for the detection of *E. lata*.

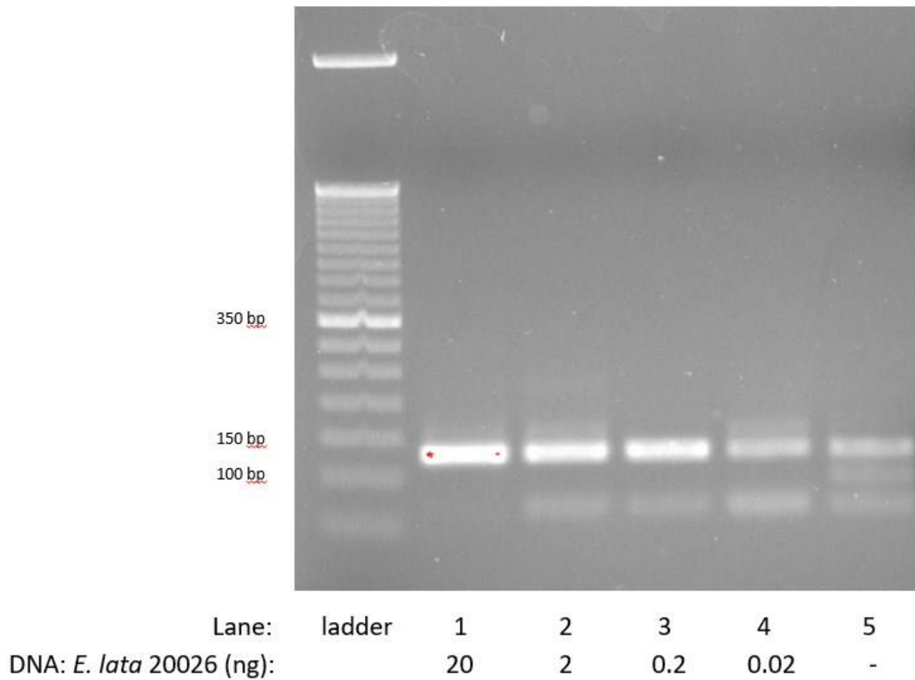


Figure 4.9. The sensitivity test of endpoint PCR of *Eutypa lata* from serially diluted target DNA. Each reaction contains 1 µL of pure *E. lata* 20026 DNA except for water control (lane 5). Target DNA amount was at 20 ng (lane 1), 2 ng (lane 2), 0.2 ng (lane 3) and 0.02 ng (lane 4). Amplification bands can be seen in the negative control lane (lane 5).

4.3.3 Real-time PCR sensitivity of *A. pullulans* and *E. lata*

4.3.3.1 Sensitivity of *A. pullulans* identification with real-time PCR

To determine whether real-time PCR may be more sensitive for *A. pullulans* detection than end-point PCR a comparative sensitivity test was undertaken. The minimum detected the amount of *A. pullulans* DNA by real-time PCR was 0.02 ng with a mean Cq value of 27.03 cycles. The accumulation curves when using water as a template were stable and did not raise in absorbance until the end of 40 cycles (Figure 4.10, Table 4.5).

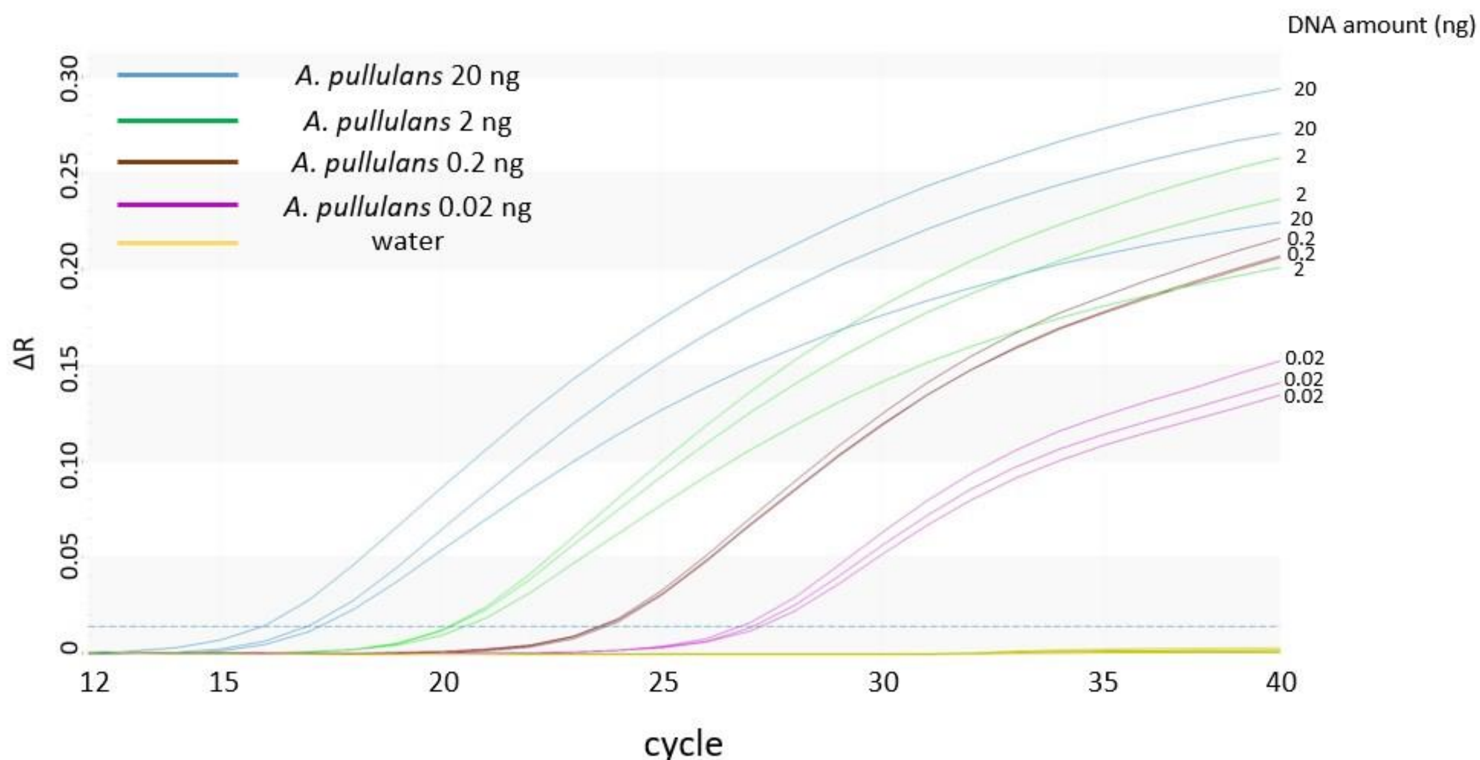


Figure 4.10. Real-time PCR sensitivity results of four amounts of *Aureobasidium pullulans* 3057 pure DNA (n=3). A Taqman probe method was used for real-time PCR to detect *A. pullulans*.

Table 4.5. Mean Cq value of *Aureobasidium pullulans* sensitivity tests by real-time PCR (n=3).

DNA amount of *A. pullulans* (ng)	Mean Cq value
20	16.73
2	20.31
0.2	23.64
0.02	27.03
water	-

4.3.3.2 Sensitivity of *E. lata* detection with real-time PCR

Real-time PCR using the SYBR Green method efficiently amplified *E. lata* and results from positive/negative templates were easy to distinguish (Figure 4.10). Amplification curves were also distinct when using a different amount of *E. lata* DNA as a template. The highest

amount of *E. lata* template DNA (20 ng) gave a curve with the lowest Cq value and lower amounts of DNA had higher Cq values as expected (Figure 4.10). At 0.02 ng amount, *E. lata* 20026 DNA was detected at a Cq value of 35.39 while the Cq value for water was 38.79, i.e. slightly over 3 cycles difference (Table 4.6).

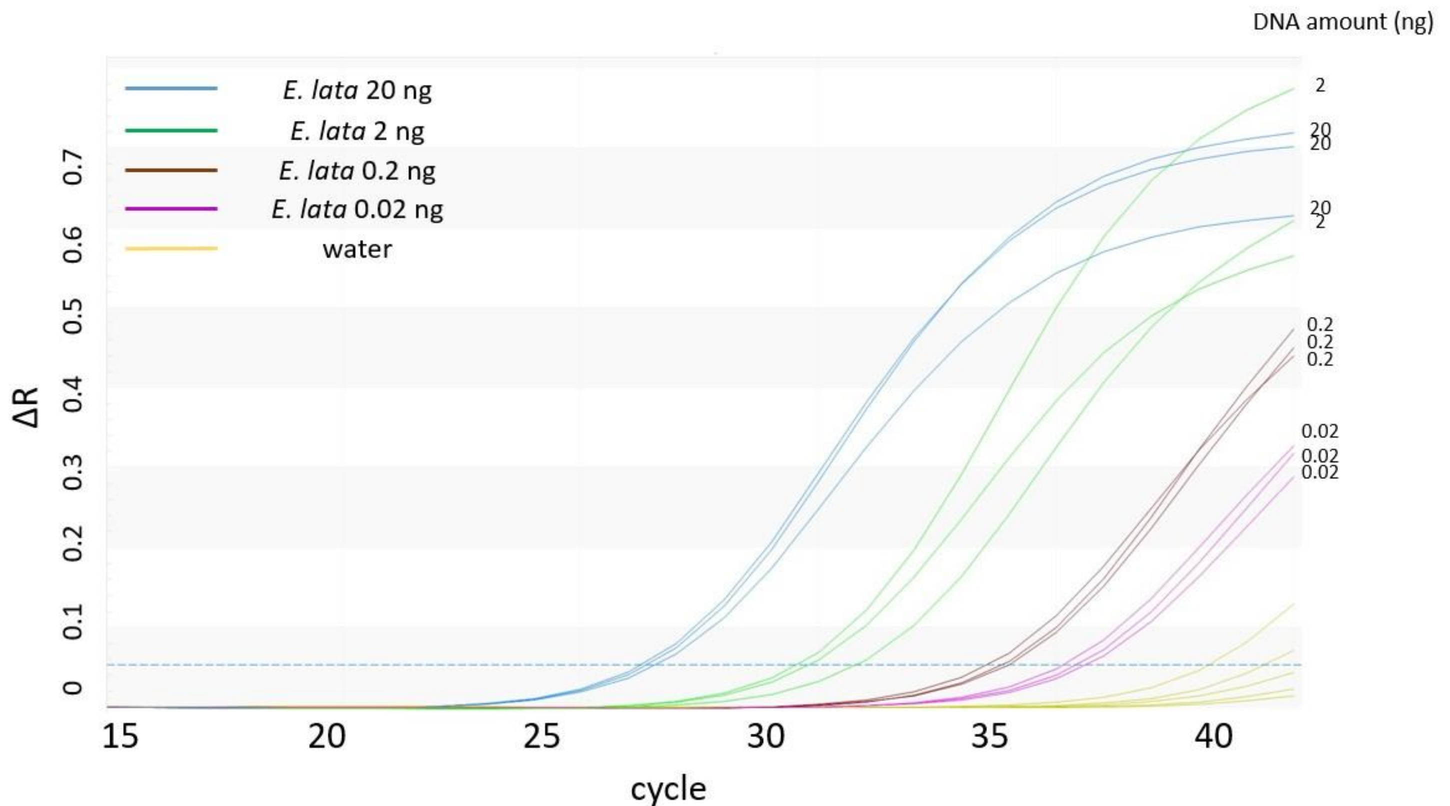


Figure 4.10. Real-time PCR sensitivity results of four amounts of *E. lata* 20026 DNA (n=3). SYBR-Green methods were used for real-time PCR.

Table 4.6. Mean Cq value of four amounts of *Eutypa lata* DNA in real-time PCR (n=3). Strong positive showed in DNA amount of 20, 2 and 0.2 ng, while DNA at 0.02 ng showed a weak positive result (ΔCq<3)

DNA amount of *E. lata* (ng)	Mean Cq value
20	26.38
2	30.02
0.2	33.78
0.02	35.39
water	38.79

Melt curves were used to distinguish weak positive results for *E. lata* DNA detection and the water controls (Figure 4.11): accumulation curves derived from the amplification of an initial amount of 0.02 ng *E. lata* DNA showed a weak peak at Tm=83.8°C, i.e. the same Tm as other stronger positives. By contrast, water controls did not show any increase in absorbance at 83.8°C. This test explained the additional bands previously produced from water control in previous tests (section 3.3.1.2) Since the water control gave no peak at 83.8°C the extra bands may be primer dimers instead of contaminating DNA. Even *E. lata* templates showed other peaks of derivative fluorescence between 76°C to 80°C, they all shared the exact same Tm at 38.8°C, which was sufficient to differentiate their presence from negative controls.

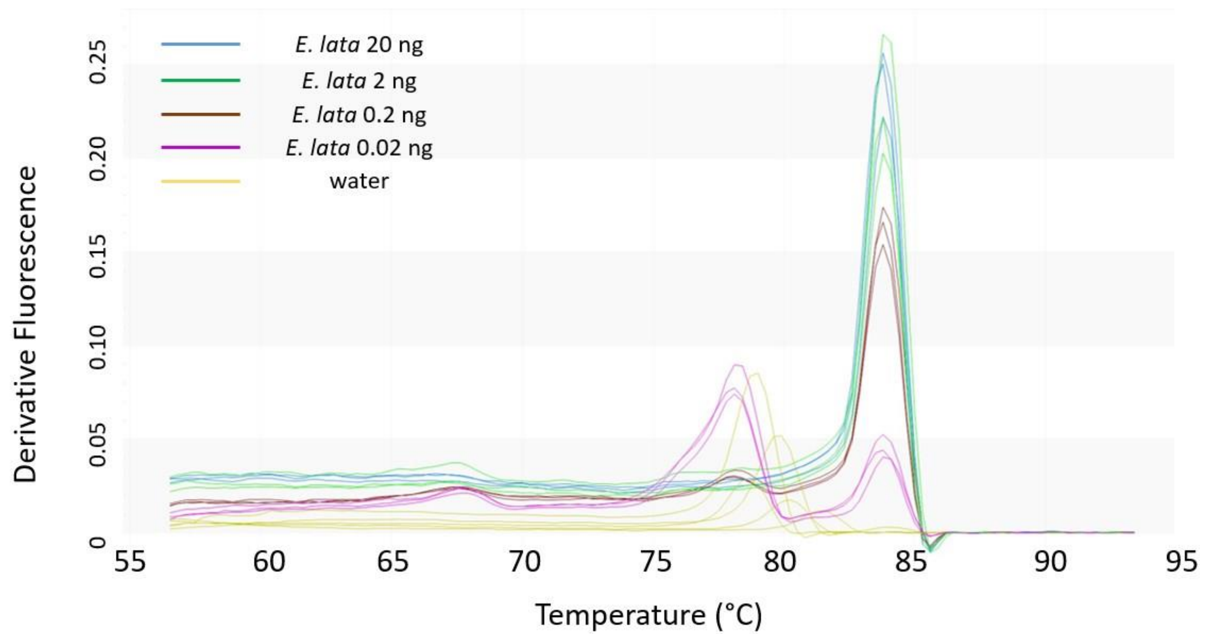


Figure 4.11. Melting temperature curves of four amounts of *Eutypa lata* 20026 DNA in real-time PCR (n=3). All four DNA amounts showed a peak of derivative fluorescence at 83.8°C though they also had peaks at other temperatures.

4.3.4 Summary of *in planta* antagonism results

This section contains specific research questions and the associated statistical significance (calculated by one-way ANOVA, 95% confidence level) generated from the comparison of each set of specific data. The results generated from the analysis of the *in planta* single and dual-inoculation tests appear as tables and associated statements that summarize the findings of the results.

4.3.4.1 Impact of fungal inoculation treatments on leaf/root presence and total stain size in canes

The impact of each fungal treatment on the presence of leaves or roots and the total cane stain length are summarised in Table 4.7.

Table 4.7. Statistical analysis of the impacts of *Aureobasidium pullulans* (four strains) and/or *Eutypa lata* (strain 20026) inoculation on grapevine biotic stress responses (leaf presence, root presence or total internal stain length). Data analysis was performed by one-way ANOVA at 95% confidence level.

Comparisons	*A. pullulans* strain	Leaf present Yes/No (p-value)	Root present Yes/No (p-value)	Total stain length Yes/No (p-value)	Comments
C1: Inoculation of *A. pullulans* vs inoculation of PDA	3057	No (N/A)	No (0.330)	No (0.297)	No statistical support
	19713	No (N/A)	No (N/A)	No (0.383)	
	20345	No (N/A)	No (N/A)	No (0.201)	
	21143	No (0.330)	No (N/A)	Yes (0.004)	*A. pullulans* results in a significantly smaller stain than PDA inoculation
C2: Inoculation of *E. lata* vs inoculation of medium only	N/A	No (0.330)	No (0.330)	No (0.194)	No statistical support
C3: Inoculation of *A. pullulans* vs inoculation *E. lata*	3057	No (0.330)	No (1)	No (0.858)	No statistical support
	19713	No (0.330)	No (0.330)	No (0.648)	
	20345	No (0.330)	No (0.330)	No (0.980)	
	21143	No (1)	No (0.330)	No (0.051)	

C4: Inoculation of *A. pullulans* with *E. lata vs A. pullulans* alone	3057	Yes (0.007)	No (0.054)	No (0.655)	Co-inoculation of *A. pullulans* strains 3057 and 19713 and *E. lata* results in more canes with leaves than inoculation of *A. pullulans* alone
	19713	Yes (0.150)	No (0.065)	No (0.776)	
	20345	No (N/A)	No (0.330)	No (0.069)	No statistical support
	21143	Yes (0.017)	Yes (0.001)	No (0.293)	Co-inoculation of *A. pullulans* strain 21143 and *E. lata* results in more canes with leaves and roots than inoculation of *A. pullulans* alone.
C5: Inoculation of *A. pullulans* with *E. lata* vs inoculation of *E. lata* only	3057	Yes (0.007)	No (0.054)	No (0.765)	*A. pullulans* 3057 results in more canes with leaves than canes inoculated with *E. lata*
	19713	No (0.555)	No (0.287)	No (0.442)	No statistical support
	20345	No (0.330)	No (0.054)	No (0.065)	
	21143	Yes (0.017)	No (1)	No (0.202)	*A. pullulans* 21143 results in more canes with leaves than *E. lata*

Statements

1. Inoculation of *A. pullulans* did not significantly affect leaf or root presence compared to canes inoculated with medium alone, and *A. pullulans* 21143 is the only strain that increased total stain size compared to canes inoculated with PDA alone (Table 4.7, C1).

2. Inoculation of *A. pullulans* alone did not affect the presence of leaves or roots or the total stain size compared to inoculation of *E. lata* alone (Table 4.7, C3).

3. Inoculation of *A. pullulans*, when co-inoculated with *E. lata*, showed a limited impact on the leaf or root presence compared to inoculation of *E. lata* or medium only. *A. pullulans* 21143 affected positively leaf presence and inoculation with *A. pullulans* 20345 was associated with increased root presence (Table 4.7, C2 and C5).

4. Dual-inoculation of *E. lata* with *A. pullulans* was associated with positive impacts on leaf existence and root existence, compared to inoculation of *A. pullulans* alone, this influence depends on the strain of *A. pullulans* inoculated. Inoculation with *A. pullulans* 3057, 19713 and 21147 were associated with increased leaf existence and *A. pullulans* 21143 was associated with increased root existence (Table 4.7, C4).

4.3.4.2 Detection of *A. pullulans* and *E. lata* from grapevine DNA using either end-point PCR or real-time PCR

End-point PCR and real-time PCR were both performed to detect the presence of *A. pullulans* and *E. lata* in inoculated grapevine canes. The comparison of the two PCR methods and their ability to detect each fungus are in Tables 4.8 and 4.9.

Statement

From Table 4.9 and Table 4.10, some conclusions on the comparison of end-point PCR and real-time PCR were listed below. In general, Real-time PCR showed increased reliability for detection of *E. lata* and higher sensitivity for *A. pullulans* than end-point PCR.

1. All canes (except for untested treatments in Group 7 to 10) were positive for *E. lata* by end-point PCR (Table 4.8, Group 1 to 7).

2. More canes were identified positive to *A. pullulans* by real-time PCR than end-point PCR when inoculated with both fungi. This phenomenon depended on the strain of *A. pullulans* that was inoculated. When co-inoculated with *E. lata*, *A. pullulans* 19713 and 20345 showed 100% positive in canes (Table 4.8, Group 3 and 4; Table 4.9, Group 3 and 4). When inoculated by itself, *A. pullulans* 19713 showed higher positive rate than other three strains (Table 4.8, Group 6 to 9; Table 4.9, Group 6 to 9).

3. More canes that were inoculated with *A. pullulans* alone were identified positive for *A. pullulans* by real-time PCR than by end-point PCR (Table 4.8, Group 6 to 9).

4. For treatment 5 (inoculated two PDA plugs) and 6 (inoculated *A. pullulans* 3057), end-point PCR showed significantly more *E. lata* present than real-time PCR, and positive bands repeatedly existed in end-point PCR water controls. Thus, the end-point PCR results were unreliable and there was no need to perform further tests by end-point PCR (Table 4.8, Group 5 and 6).

Table 4.8. Grapevine canes identified with *Aureobasidium pullulans* and *Eutypa lata* by end-point PCR and real-time PCR. The * indicates some DNA samples were excluded due to either storage tubes were broken or there was no sufficient amount of DNA for amplification in the extraction. The ¤ indicates test results were invalid due to positive results in the water control.

	Fungal Inoculation		**Fungal positive canes by end-point PCR**		**Fungal positive canes by real-time PCR**		**Were all *A. pullulans* positive canes by end-point also positive by real-time?**
Group number	*A. pullulans*	*E. lata*	*A. pullulans*	*E. lata*	*A. pullulans*	*E. lata*	
1	3057	20026	4/7*	7/7*¤	5/7*	7/7*	No
2	21143	20026	2/9*	9/9*¤	3/9*	9/9*	Yes
3	19713	20026	0/9*	9/9*¤	9/9*	9/9*	No positive in real-time
4	20345	20026	0/10	10/10¤	10/10	10/10	No positive in real-time
5	-	-	4/10	10/10¤	6/10	1/10	Yes
6	3057	-	0/9*	9/9*¤	4/9*	1/9*	No positive in real-time
7	21143	-	0/10	10/10¤	5/10	0/10	
8	19713	-	0/10	N/A	7/10	0/10	
9	20345	-	0/9*	N/A	4/9*	1/9*	
10	-	20026	0/9*	N/A	2/9*	9/9*	
Positive band in water control?			0/10	7/7	0/10	0/10	All end-point PCR showed *E. lata* positive bands in water control.

Table 4.9. Comparison of PCR detection method (real-time and end-point) on total fungal identification and significance. Statistical analysis was performed by one-way ANOVA at 95% confidence level. The * indicates real-time PCR found more *A. pullulans* than end-point PCR. The [¤] indicates end-point PCR found more *E. lata* than real-time PCR even in the water control. The [Δ] indicates clear bands were repeatedly generated in the water controls.

Group No.	Treatment		Impact on *A. pullulans* detection: Yes/No (p-value)	Impact on *E. lata* detection: Yes/No (p-value)	Comments
	A. pullulans	*E. lata*			
1	3057	20026	No (0.096)	No (1)	No statistical support
2	21143	20026	No (0.283)	No (N/A)	
3	19713	20026	Yes* (less than 0.0001)	No (0.332)	Real-time PCR significantly increased detection of *A. pullulans* 21143 and 19713
4	3057	20026	Yes* (0.027)	No (0.150)	
5	-	-	No (0.748)	Yes (less than 0.0001)[¤]	Real-time PCR showed significantly less *E. lata* positive results in group 5 and 6, also significantly increased *A. pullulans* detection in group 6 to 9.
6	3057	-	Yes* (0.035)	Yes (Less than 0.0001)[¤]	
7	21143	-	Yes* (0.007)	N/A[Δ]	
8	19713	-	Yes* (0.001)	N/A[Δ]	
9	20345	-	Yes* (0.028)	N/A[Δ]	
10	-	20026	No (0.150)	N/A[Δ]	

4.3.4.3 Comparison of fungal detection in different treatments by real-time PCR

Due to uneven DNA sample numbers in different treatments as a result of some low yields of DNA from grapevine canes, it was not appropriate to perform ANOVA tests to compare differences of the fungi detected in different treatments by real-time PCR. Instead, a simple statistical study was undertaken by directly comparing the ratio of positive samples in total DNA samples. The summary of results is as follows:

1. Except for *A. pullulans* 21143, all other three *A. pullulans* strains showed higher *A. pullulans* presence when co-inoculated with *E. lata*, compared to canes inoculated with *A. pullulans* only and canes inoculated with two PDA plugs (Table 4.8, Group 1 vs 6, 2 vs 7, 3 vs 8, 4 vs 9).

2. When treated with *A. pullulans* only, *A. pullulans* 19713 was the only strain that showed higher *A. pullulans* presence than canes inoculated with two PDA plugs (Table 4.8, Group 5 to 9).

3. Co-inoculation of any of the four *A. pullulans* strains along with *E. lata* significantly increased the incidence of *E. lata* presence compared to canes inoculated with *A. pullulans* alone (Table 4.8, Group 5 to 1 to 4, 6 to 9).

4. Co-inoculation of *A. pullulans* together with *E. lata* did not reduce *E. lata* presence, compared to canes inoculated with *E. lata* alone (Table 4.8, Group 1 to 4, 6 to 9).

4.4 Discussion

Multiple studies focussing on the interaction between *A. pullulans* and a pathogen associated with a GTD have occurred over the last 10 years (Dimakopoulou, Tjamos et al. 2008, Rühmann, Pfeiffer et al. 2013, Pinto, Custodio et al. 2018, Don, Schmidtke et al. 2019). However, to my knowledge, no studies have considered applying *A. pullulans* as BCA against *E. lata*. This study represents the first approach into the antagonism of *A. pullulans* and *E. lata* within both *in vitro* and *in planta* conditions.

4.4.1 Leaf and root presence

4.4.1.1 Shade house position

An initial finding from the visual observations of treated canes was that most canes with leaves and roots present were located in the bottom-left corner (Figure 4.4). Similarly, canes inoculated with both *A. pullulans* and *E. lata* grew leaves; *A. pullulans* 3057 and *E. lata* (treatment 1) showed 41.6% (5/12) leaf presence and those inoculated with *A. pullulans* 21143 and *E. lata* (treatment 2) showed 53.8% (7/13) leaf presence. Likewise, co-inoculation of *A. pullulans* 3057 or 21143 with *E. lata* (treatments 1 and 2, respectively) expressed high root presence. Importantly, most canes subjected to treatment 1 (41.6%, 5/12) or treatment 2 (46.1%, 6/13) were located in the bottom left area of the shade house as depicted in Figure 4.4.

The above results raised the question of whether this uneven leaf and root presence was related to the location of the pots within the shade house or the inoculation. The bottom left represented within Figure 4.4 was located in the same part of the shade house, because all inoculation pots were small and placed close. In the present study, environmental conditions were managed as similarly as possible to all canes. All canes were closely placed in six plastic trays so that the light, temperature, irrigation were as even as possible such that the experiment would test treatment of the canes rather than shade house growth position.

It is tempting to conclude that co-inoculation of *A. pullulans* 3057 or 21143 with *E. lata* results in an increase of leaves and roots, compared to canes inoculated with *A. pullulans*

alone, *E. lata* alone or two PDA plugs. However, the clustering of the canes with leaves and roots suggests that at least one other variable had a strong influence on the results. This may be position within the shade house or even the source canes used for the experiment. If the source canes were not randomly distributed among the treatments then this may result in the uneven results. Likewise, in a future repeat experiment, each treatment should be randomly distributed across the shade house area to account for any position effect.

4.4.1.2 Correlation of roots and leaves on canes

With the combining leaf and root presence together, canes inoculated with PDA only showed no leaves or roots. This result is different from our prediction. Instead, the co-inoculation of two fungi promoted plant growth compared to canes inoculated with PDA only, *A. pullulans* only or *E. lata* only. This surprising results suggested that the treatment of two fungi into canes may have triggered some mechanisms in the cane that enhanced growth status. By contrast, the PDA only treatment seems to have provided a nutrient source in the absence of competing fungi on which existing fungi within the cane may have gained benefits that supported their proliferation (see section 4.3.1.2 that describes the stain length when inoculated with PDA only).

4.4.1.3 Impact of time post inoculation

Only 22/122 canes had grown leaves and 17/122 had grown roots by the time this study was analysed at 83 dpi. In another study that involved inoculation of *E. lata* alone or in combination with other fungi, an extended growing time of up to 18 months was provided (Camps, Kappel et al. 2010). In the research of Camps and colleagues (2010), even after this long incubation period, grapevine canes still showed a significant decrease in leaf and shoot growth with a larger proportion of *E. lata* infected samples growing shorter steams with necrosis on leaves than the control uninfected plants. A longer incubation period for the experiment in my research may have also provided quantitative data on leaf and shoot growth rather than the simple presence/absence approached used after 83 dpi.

4.4.2 Grapevine cane stain analysis and potential plant defense mechanisms

4.4.2.1 The impact of inoculation on cane stain size

The measurement of stain sizes in inoculated canes provides us with information about plant pathogen responses that were not involved in the *in vitro* set of experiments.

Canes inoculated with PDA-only had the highest stain incidence and largest average stain size. At least two reasons may explain this phenomenon. First, the cane samples used for the inoculation study may have been infected with *E. lata* and /or other stain-inducing phytopathogens prior collection or at the time of inoculation. The cane samples were collected from one-year-old shoots growing on healthy-looking grapevines. The selection of young canes was made to reduce the likelihood of existing infection that might be more likely to be higher on older shoots. However, we cannot guarantee that the collected canes were disease-free. Second, we performed the inoculation in a PFR Blenheim laboratory that is used for GTD isolations. Therefore, there is potential for unintended infections from spores in the air that may have entered into wounds and developed over the incubation time.

Regardless of the origin of infection, the inoculated PDA medium acted as a “catalyst” rather than an innocuous negative control that likely provided phytopathogenic microbes within the canes extra nutrients that supported their rapid growth and pathogenicity resulting in many and large stains. Thus, the use of the nutrient-rich PDA medium encouraged opportunistic pathogen growth inside the canes resulted in the activation of plant defense pathways and stains. More appropriate negative controls would have included a series of treatments including no hole drilled, a hole drilled with no medium inserted, a hole drilled with a low nutrient medium (water agar) inserted, and also the control that we used, the insertion of PDA into the drilled hole. Such a series of ‘negative control’ treatments would be certain to provide a reliable negative control against which to measure the fungal inoculum treatments. This suggested series of negative controls might also assist in teasing apart which aspect of the inoculation process contributes to staining development.

*A. pullulansE. lata*When inoculated in a co-infection with *E. lata*, only *A. pullulans* 21143 acted to reduce stain size compared to samples inoculated with PDA only. Also, there was

no statistical evidence the co-inoculation of two fungi can significantly reduce total stain size than *E. lata*-only treatment. This indicates *A. pullulans* treatment did not protect grapevine canes in a way we expected. There were two potential explanations for these results. First, *A. pullulans* may not protect *A. pullulans* in reality and we cannot confidently eliminate this possibility. Secondly, our experimental design did not provide a suitable environment to demonstrate the protective effects of *A. pullulans*. Some related factors might be inappropriate indicator of stain size, insufficient time for cane growing, uneven amount of inoculated fungi into canes or inner micro-organisms disrupted our study. More detailed discussion for unexpected results in section 5.2.

4.4.2.2 The physiology of grapevine cane or trunk stains

The presence of a stain in a grapevine cane (or trunk) is an indicator of plant disease either previously or currently associated with an active, disease-promoting pathogen or pathogens. Thus, a stain does not necessarily indicate the current status of fungal presence (Sosnowski, Lardner et al. 2007). Typically, cane or trunk stains represent previous development of pathogens that have triggered a plant response resulting plant cell death and, in turn, a degree of pathogen death. Specifically, the trunk stain in GTD is a piece of evidence that plant defense system has been triggered. Typically, fungal pathogens cannot be identified in cane or trunk stains (Mundy and Manning 2011).

Apart from stains, there are more physiological responses occurring in affected trunk vascular tissues. The secondary xylem of grapevines has a ladder-like structure surrounded by living xylem (Mullins, Bouquet et al. 1992). The xylem has a major defense activity in the form of tyloses that occlude the vessel lumen (Gómez, Báidez et al. 2016). Scanning electron microscopy shows some newly formed tyloses that occlude the root vessel lumen when inoculated with *E. lata* (Figure 4.12). This xylem occlusion restricts water flow towards the infected area and reduces fungal mycelium growth and thus slows down disease spread speed within the plant. Although these vascular occlusions might help grapevines defense against the challenge of a pathogen, it also creates visual symptoms of the battle that has taken place and can count as a more severe disease status than it is at the time of viewing.

However, a secondary impact is reduced water flow beyond the stain (or canker) and this may result in severe symptom expression.

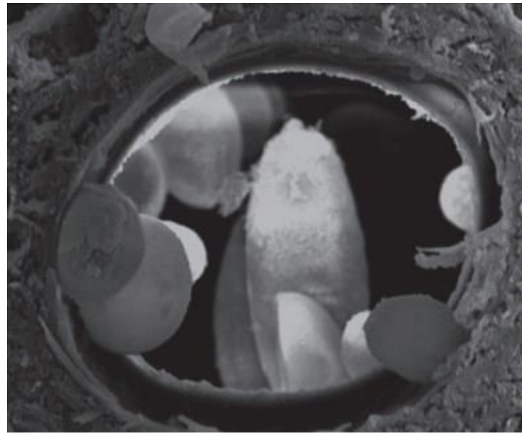


Figure 4.12. Scanning electron micrograph of grapevine cane cross-section. Tyloses at varying degrees of development in a xylem vessel (Gómez, Báidez et al. 2016). These tyloses may eventually block the entire xylem vessel in the infected area, causing more severe symptoms.

The mechanisms behind pathogen invasion, plant defence and impact on a plants performance can be complicated. Plant defense systems can be triggered by a variety of factors. Plant defense responses include producing reactive oxygen agents, synthesis of phytoalexins (particularly resveratrol and viniferin), repair of plant cell walls and production of pathogenesis-related proteins production (Aziz, Trotel-Aziz et al. 2006). Grapevines can secrete phytoalexins in response to fungal infections to protect themselves. Resveratrol and viniferin are two major phytoalexins to suppress fungal pathogens. They were synthesized by grapevine trunks when inoculated with GTPs such as *E. lata*, *B. cinerea* (Stempien, Goddard et al. 2017). These secondary metabolites play roles in inhibiting spore germination, fungal penetration and disturbing pathogen growth (Stempien, Goddard et al. 2017). That study identified a significant inhibitory ability of viniferin on *N. parvum*, which is another critical causal agent of GTD. Viniferin can also inhibit the growth of *E. lata* by reducing its colony radius within *in vitro* conditions (Alessandro, Di Marco et al. 2000). Intriguingly, viniferin is one potential beneficial compound in some wines for its anti-oxidation effects (Pawlus, Waffo-Téguo et al. 2012). Some studies claim that a combination of viniferin and other stilbenes in wine can decrease the severity of many chronic diseases (Pawlus, Waffo-Téguo et al. 2012).

Stained canes in this study normally had a strong and pleasant smell that issued from their discoloured tissues; it smelled like a crispy wine. It is unknown what the source of this smell was but it did not smell similar to either *A. pullulans* or *E. lata* cultures. Since this smell only came from stained canes, it was possible that it was the result of pathogen triggered plant defense response.

4.4.3 Fungal presence in inoculated samples

4.4.3.1 Optimising PCR protocols for identification of *A. pullulans* and *E. lata*

Optimising the PCR protocols for the *in planta* identification of *A. pullulans* and *E. lata* was a time-consuming part of the entire thesis research. Initial trials to amplify *E. lata* and *A. pullulans* by end-point PCR showed significantly different results depending on the target organism. As described in Chapter 3.2, end-point PCR protocols were applied for fungal identification both *in vitro* and *in planta*. From the 62 effective samples out of 64 cane samples, 100% of them were classified by end-point PCR as *E. lata* positive but none of them was identified as *A. pullulans* positive by end-point PCR, even though they were all inoculated with both *E. lata* and *A. pullulans*. The likelihood of all samples being infected by *E. lata* was possible but unlikely. Furthermore, these results did not reflect the predominant biomass of *A. pullulans* from the NGS data (section 2.3.4), and the absence of *A. pullulans* in all samples even after its inoculation also seemed unlikely. Consequently, further optimising of PCR methods for *E. lata* and *A. pullulans* was required.

To improve the sensitivity and specificity of fungal detection by PCR from inoculated canes, a series of trial experiments were undertaken (section 4.3.2.2). These assays were designed to answer two research questions:

1) What sensitivity does the PCR have for detection of *A. pullulans* and *E. lata*?
2) What has caused negative detection of *A. pullulans* from grapevine cane samples? How to improve the detection sensitivity of *A. pullulans* in grapevine DNA?

The experiments identified that grapevine DNA had an inhibitory effect resulting in low sensitivity of *A. pullulans* DNA; the limit of detection was 10 ng. Many studies have found that grapevines typically contain compounds that inhibit PCR activities (Dovas and Katis

2003, Osman and Rowhani 2008). Inhibitory effects of plant components in PCR is often caused by acidic polysaccharides such as carrageenan, gum ghatti and gum karaya (Pandey, Adams et al. 1996). Although the CTAB method is a relatively quick and efficient DNA extraction method from plants it still needs to be optimised for grapevine DNA extraction so as to remove grapevine polysaccharides effectively (Porebski, Bailey et al. 1997). The CTAB method used in this study was not specifically optimised for grapevine trunk samples. Typically when the extracted DNA yielded good total yield (at or above 20 ng/µL), they were believed sufficient to perform PCR based detection methods as advised by Pratap Vanga, PFR scientist, Lincoln.

In terms of the second question, one potential solution explored to counter the inhibitory compounds present in the DNA was the addition of BSA (in chapter 3.2.5.5) to bind and thus deactivate the PCR-inhibitory compounds. Although adding BSA was effective the end-point PCR detection sensitivity of *A. pullulans* was still not as good as real-time PCR (section 4.3.2.1) and showed many false-negative results in detecting real grapevine DNA (section 4.3.4.2). Thus the statistical analysis was based on real-time PCR results.

4.4.3.2 Molecular detection of fungi *in planta* by end-point PCR and real-time PCR

Chapter 4.3.4 revealed fungal presence by both end-point PCR and real-time PCR. Real-time PCR was more specific and sensitive than end-point PCR in detecting both fungi. Thus, all data comparisons were subsequently based on real-time results.

The application of real-time PCR has accelerated our experiment speed by its fast speed and high reliability. Real-time PCR does not require gel electrophoresis, which saved 30-40 mins per plate. A faster pace also allowed us to optimise amplification protocols within a shorter time than end-point PCR. In addition, real-time PCR helped to avoid unwanted bands when detecting *E. lata* in grapevine DNA samples (section 4.3.4.3) and showed correct presence of *A. pullulans*.

End-point PCR also has advantages. After gel electrophoresis, we are able to observe product size by comparing with appropriate DNA ladders. This was an easy method to illustrate if correct product was amplified with these primers. Also, we could sequence positive band to further make sure we got correct product.

4.4.3.3 The impact of inoculation combinations and fungal species presence or absence

A. pullulans strains were persistent within inoculated canes (treated by two fungi or *E. lata* only) but the presence of *A. pullulans* generally failed to reduce the presence of *E. lata*, with no exception. However, the co-inoculation of *E. lata* has increased the presence of *A. pullulans* 3057, 19713 and 20345 (section 4.3.4.1, Table 4.8).

A. pullulans showed positive in 6 of 10 canes treated with PDA only (section 4.3.4.2, Table 4.8), this ratio is even higher than canes in group 2 (*A. pullulans* 21143 and *E. lata*) and 10 (*E. lata* only). This indicated our cane samples may already have a high presence rate of *A. pullulans* prior to inoculation. It would be beneficial for us to run the sequencing of contained species in cane samples before doing any treatment (section 5.4). A larger number of replicates than our study would answer if inoculation of *E. lata* has a suppression of inner *A. pullulans* contained.

The results stated above were not easy to rationalise however two possible explanations were developed. Firstly, the interaction between two fungi may have favoured the growth of *A. pullulans*. This indicates competition is probably not the only activity involved within two fungi. Secondly, *A. pullulans* may have also been suppressed by the plant defense system despite *A. pullulans* commonly being regarded as an endophyte. The existence of *E. lata* interfered plant responses to some extent, thus it helped *A. pullulans* to acquire more niches to develop. Finally, enzymes secreted by *E. lata* could also degrade plant tissue and provide extra nutrients to *A. pullulans*. Thus there is the probability that *E. lata* can promote *A. pullulans* colonizing grapevine cane tissues. It was very interesting to see phytopathogen (*E. lata*) has helped potential BCA (*A. pullulans*) to colonize grapevine tissue, more discussion about this were in section 5.3.

Since there was a low risk of *E. lata* in canes prior to fungal inoculation (low *E. lata* rate in PDA-only treated canes, section 4.3.4.2), the high stain rate in PDA-treated canes may fit other illustrations. By adding extra nutrients (PDA medium) into cane wounds, other pathogens in canes acquired opportunity to grow and this induced plant response. Because

E. lata was the only tested fungal pathogen by real-time PCR, other micro-organisms might be the reason for cane stains.

4.4.3.4 The impact of fungal species present and cane physiology

It is not easy to co-relate fungal presence with cane physiology. There are different factors of grapevine health status studied in this research: the presence of leaves and roots, stain size (upper, lower and total). And we did not find a proper statistical method to link all these factors in one model. Because the presence of fungal species, leaves/roots, total stain size were different variables, we had a simple comparison among them instead of statistical method.

Treatment 4 (*A. pullulans* 20345 with *E. lata*) has the highest number of stained canes (10/12) and the largest average stain size (4.40 cm). And all canes (10/10) were identified positive to both fungi. However treatment 5 (PDA only) has the second-highest stain rate (10/12) and second-largest average stain size (3.33 cm), but canes showed medium positive rate to *A. pullulans* (6/10) and low presence of *E. lata* (1/10). We cannot generate useful support that fungal presence by real-time PCR has any indication to cane physiology.

4.4.4 Problems encountered

DNA isolation from grapevine cane samples was difficult and time-consuming and resulted in only low and inconsistent yields of DNA. The physical nature of the cane material was hard resulting in insufficient and inconsistent homogenisation by mortar and pestle under liquid nitrogen followed by bead ruptor treatment. Some (13/122 cane samples) of the extraction yield was not enough for molecular study. A method that is more efficient in time, energy and DNA yield would produce more consistent and reliable results compared with the method used. Pratap Vanga and Dion Mundy used drill during trunk collection, which resulted in near-powder tissues. This made the following homogenisation easy and the yield of DNA can be predicted.

The DNeasy® Plant Mini Kit was trialled for DNA isolation from grapevine cane samples but the yield was poor. The spin tubes from the extraction kit were too small to fit sufficient

plant material. The CTAB method gave better and more consistent yields than the kit and, as such, was used for all DNA extractions.

The discrimination between positive and negative results for *A. pullulans* and *E. lata* from DNA samples was difficult by end-point PCR but was more easily achieved by real-time PCR. These issues were expressed in two different ways for *A. pullulans* and *E. lata*. For *A. pullulans*, the end-point PCR bands were inhibited by grapevine materials (see section 4.2.6.5), that led to many false-negative results. By contrast, when using end-point PCR bands *E. lata* identification showed positive bands in almost every reaction including water controls, making it impossible to identify true positives among the false-positive results.

The two issues described above were both resolved by real-time PCR. For *A. pullulans*, the sensitivity was increased from 0.2 ng using end-point PCR to 0.02 ng using a Taqman probe-based real-time PCR method (section 4.2.6.4 to 4.2.6.5). For *E. lata*, the specificity was increased using SYBR-Green based real-time PCR and HRM analysis that enabled the distinction between true PCR products and background products that were likely primer dimers.

In a trail PCR experiment for both *A. pullulans* and *E. lata* from real grapevine DNA, the results suggested a more sensitive alternative approach was required (section 4.2.6.4) The real-time PCR described above gave better sensitivity and specificity than endpoint PCR. However, even using the improved real-time PCR protocols, comparisons between samples could were not fully optimised. Due to the time limitation of my project, the grapevine reference gene was not developed to quantify and compare fungal DNA amount in each grapevine sample. Instead, a standardized amount of grapevine DNA was used to compare between samples and to standardise between different yields from the CTAB extraction. This method resulted in providing only the presence or absence of *A. pullulans* and *E. lata* rather than their relative abundances.

4.4.5 Summary of results and future directions

In brief, the results of this study can be summarised by several statements.

Co-inoculation of *A. pullulans* (3057 and 21143) and *E. lata* increased the presence of leaves and roots in canes compared to canes treated with *E. lata* only, *A. pullulans* only and PDA only (section 4.3.1).

Co-inoculation of *A. pullulans* (20345 and 3057) with *E. lata* significantly increased total stain size in trunk compared to canes treated with the same *A. pullulans* strains (section 4.3.1).

Real-time PCR can identify the presence of *A. pullulans* and *E. lata* in grapevine DNA with good sensitivity and avoid negative effects of PCR inhibitors (section 4.3.2).

Co-inoculation of *A. pullulans* with *E. lata* did not show significant protection under current study methods.

In summary, future studies on the impact of *A. pullulans* and *E. lata* single or dual-inoculations on grapevine canes would include the following (more detailed improvements were in section 5.4):

1. Use a drill to collect cane samples to avoid difficult homogenisation step in section 4.2.6.1 and obtain good yield of DNA.

2. Sequence grapevine DNA prior to inoculation, thus we could understand fungal species within inner tissue and enhance understanding other species' impact (section 4.3.1.2).

3. Perform experiments on live grapevine trunks instead of cutting canes and grow in shade house. This would help to mimic real growing condition thus it can reflect fungal protective effects better (section 4.2.1).

4. Inoculate fungi with water agar to replace PDA into grapevines. This can minimise effect of additional nutrients so neither of inoculated fungi nor inner fungi would grow too fast (section 4.2.2).

5. In assessing the fungal presence, use grapevine reference genes to identify the relative abundance of each fungal DNA across the samples rather than simply their presence or absence (section 4.2.6).

5. General Discussion

There are many gaps in knowledge regarding the interaction between *A. pullulans* as a BCA and grapevine health. GTDs cause severe yield reduction in New Zealand and around the world, resulting in significant economic loss. *A. pullulans* is applied commercially to decrease negative effects of pathogens in crops (e.g. grapevine, strawberry and kiwifruit). However, the diversity of vineyard management systems and causal agents that result in GTDs means that it is difficult to find a single GTD management method that is effective and can be used in a range of situations. Although GTDs are common grapevine disease current management systems are limited and include reducing new infections through the protection of pruning wounds combined with identifying any infected and no-productive vines followed by vine replacement. This research project sought to contribute to addressing the following key knowledge gaps:

1. Determine the impact of *A. pullulans* on *E. lata* abundance in commercial vineyards over years using *in silico* data;

2. Determine how the abundance of *A. pullulans* and *E. lata* affect GTD symptoms in vineyards;

3. Determine whether there is any inhibitory impact of *A. pullulans* on *E. lata* colony growth *in vitro*;

4. Determine how *A. pullulans* affects *E. lata* movement and GTD symptoms *in planta*.

It is hoped that this study can contribute to the development of biological control of GTDs using *A. pullulans*. The combination of *in silico*, *in vitro* and *in planta* studies was used to address the knowledge gaps above however further research is required to determine the potential of *A. pullulans* for GTD management, especially *in planta*. Such *in planta* evidence would be required prior to consideration of any commercial development or application.

5.1 Summary of results from this research

This study uncovered *A. pullulans* can inhibit the growth of *E. lata* colonies on PDA plates and restrict *E. lata* fungal abundance in commercial vineyards. However, there was no statistical evidence showing co-inoculation of *A. pullulans* and *E. lata* can increase the presence of leaves and roots or reduce total stain size compared to canes inoculated with *E. lata* only.

In silico, we identified *A. pullulans* abundance varied among management systems and grapevine cultivars. Future managed vines and Sauvignon blanc vines had a lower abundance of *A. pullulans* than vines under contemporary management and Pinot Noir. But *E. lata* seemed not affected by these factors. *A. pullulans* reduced *E. lata* abundance in grapevines, but neither *A. pullulans* nor *E. lata* has a connection to GTD symptoms.

In vitro, results were as predicted: all *A. pullulans* inhibited *E. lata*, and this effect was unique from non-BCA species (*B. cinerea*). *A. pullulans* had the highest reduction of *E. lata* colony radius and was also the least reduced *A. pullulans* strain by *E. lata*.

In planta tests results were different from our prediction: *A. pullulans* did not express sufficient protection against *E. lata* infection. In fact, co-inoculation of two species have increased the presence of leaves and roots. Canes treated with PDA only showed the highest total stain size among all groups, and we did not find an obvious link between fungal presence and cane health status under current methods.

5.2 Vineyard management effects on microbiome-particularly on *A. pullulans* and *E. lata*.

The research in Chapter 2 identified factors that affect the fungal abundance of *A. pullulans* and *E. lata*, also studied whether *A. pullulans* or *E. lata* had any impact on GTD symptoms. The analysis revealed a different abundance of *A. pullulans* and *E. lata* under different management methods and grapevine cultivars (section 2.3.4 and 5.1). There are a few explanations of these results as discussed below.

5.2.1 Impact of groundcover on soil and grapevine microbiota

There are distinct differences in groundcover species and abundance between future and contemporary managed vineyards and this may differentially affect the microbiome of grapevines in vineyards under each management system (Raw V and Waihape S 2019). In vineyards, soil normally serves as a reservoir of micro-organisms and affect microbiota in upper-ground tissue of grapevine (Zarraonaindia, Owens et al. 2015).

Vineyards with less herbicide application normally have a unique bacterial microbiome in soil from contemporary managed vineyards (Chou, Heuvel et al. 2018). This phenomenon has two fundamental explanations: 1. Herbicide changes groundcover plant species (or eliminated them entirely), thus altered the bacterial/fungal micro-organisms selectively associated with those groundcover plants (Dias, Dukes et al. 2015). 2. Herbicide directly affects micro-organisms, as discussed in section 2.4.4. Our study found a low fungal similarity between vineyards under different managements. One study gap of our study is we have not done any identification of microbiome in the soil of vineyard however, this being undertaken in the wider Vineyard Ecosystems programme with which my research aligns. Understanding the soil microbiome in the future and contemporary vineyards will provide information about the microbial environment "pool" in which the grapevines are growing in the commercial vineyard settings.

5.2.2 Effect of grapevine cultivars

Grapevine cultivar is another potential reason for fungal abundance. Sauvignon blanc vineyards have less *A. pullulans* than Pinot noir. Grapevines can facilitate soil microbiota by actively secreting anti-biotic compounds (Compant, Mitter et al. 2011). Hence, the grapevines cultivar can be reservoirs that naturally influence its microbiota. Grapevine cultivars may normally have diversity in the microbiome within different cultivars (D'Amico, Candela et al. 2018). Grapevines can "select" rhizosphere and endosphere micro-organisms through plant-soil interaction (Marasco, Rolli et al. 2018). This phenomenon was mainly generated by different rootstock cultivars of hybrid grapevines (D'Amico, Candela et al. 2018). We have no current interpretation on why Pinot Noir has a higher abundant of *A. pullulans* than Sauvignon blanc, and this did not significantly affect GTD symptoms (section 2.3.4).

5.2.3 Effect of Abiotic factors

Another factor affecting fungal abundance is weather conditions. Environmental conditions are normally influential factors on phytopathogen transmission or development. Rainfall, relative humidity, temperature and wind speed all contribute to fungal spore release, either each species separately or as a complex (van Niekerk, Calitz et al. 2010). The temperature has a strong effect in coordinating endophytic components (Campisano, Albanese et al. 2017). Endophytes component in canes and trunks are more sensitive to temperature variation than roots.

We did not analyze the abiotic conditions over each year (this is undertaken in the wider Vineyard Ecosystems programme) and it would add more variables to our symptoms-fungal microbiome model. Of note, both the Sauvignon blanc and Pinot noir analysed in Chapter 2 were from the Marlborough region where the weather can create microclimates. Whether this is the sole cause of the cultivar differences observed in the data is yet to be determined but seems unlikely. One crucial method is to managing wounds: when cutting is not practical on cankers, use a blow torch to dry all exudate in the wound and apply copper sprays. This blocks the predominant entrance on the grapevine and prevents potential infection of airborne phytopathogens (PSV 2017).

In addition, it is impractical to change weather conditions in a region to control GTDs but growers could choose to undertake appropriate activities under different weather, e.g. avoid pruning in humid weather or rainy weather as recommended (Gramaje and Armengol 2011). In some crops, the production system is altered to grow vines under cover, e.g. some kiwifruit orchards in New Zealand, so as to slow *Pseudomonas syringae pv actinidiae* (PSA) infection.

5.2.4 Grapevine nurseries affect microbiota

Microbiota can be a reliable and persistent signature of grapevine origins and environmental conditions (Mezzasalma, Sandionigi et al. 2017). Most grapevine plants in New Zealand were initially propagated in grapevine nurseries, thus the microbiota in vineyards are most likely highly influenced by the nursery environment. The grafted grapevine standard established by the New Zealand Winegrowers guards against new infection of GTDs. It contained a wide range of management protocols including virus eliminating, quality testing, vine health monitoring and many detailed regulations (NZW 2019).

However, the science behind how to manipulate a microbiome structure so that it is GTP free or poorly represents these pathogens is currently lacking. Grafted plants play roles for the transmission of micro-organisms between nurseries and vineyards. The source of rootstock and scion wood is critical to creating a grafted grapevine that is of the highest health. Furthermore, it would be useful if we could obtain nursery source data to track fungal movement among vineyards.

5.3 Can *A. pullulans* inhibit *E. lata* in grapevines?

One predominant purpose of this study was to demonstrate the antagonistic effects of *A. pullulans* on *E. lata*, to ultimately uncover whether *A. pullulans* has potential as a BCA against the GTD Eutypa. Our *in silico*, *in vitro* and *in planta* studies related the antagonistic research in separate chapters. The inhibitory efficacy of *A. pullulans* varied in each study. *A. pullulans* was negatively associated with *E. lata* as assessed *in silico* based on NGS data that included the two species, and it was antagonistic to *E. lata* growth *in vitro*, but showed only limited inhibitory effects in cane inoculation tests.

In vitro tests also brought interesting consequences different from our prediction: The inhibitory effect of *A. pullulans* on *E. lata* colony radius depends on *A. pullulans* strain (section 3.3.2), also *A. pullulans* was inhibited by *E. lata* at the same time. In addition, although *A. pullulans* 20345 was the slowest-growing strain among all isolates, it can slightly change *E. lata* colony shape prior to direct surface contact.

In planta, A. pullulans does not significantly increase the existence rate of leaves or roots, at least in treated samples. In addition, average stain sizes were slightly reduced when both fungi were inoculated comparing to blank groups.

Co-inoculation of *A. pullulans* and *E. lata* did not reduce the presence of *E. lata*, compared to canes inoculated either fungus alone. The results indicate that our current study cannot sufficiently support our hypothesis: *A. pullulans* can reduce GTD symptoms caused by *E. lata* in grapevine canes.

Apart from *A. pullulans*, many other fungal or bacterial strains were demonstrated to have potential inhibitory impacts on *E. lata* or other infections in grapevines. *Bacillus subtilis* is one of the earliest identified bacterial BCA against *E. lata* and other fungal infections (Ferreira, Matthee et al. 1991). Ethanol extraction of *B. subtilis* also expressed significant inhibitory effects *in vitro* (Leifert, Li et al. 1995).

Overall, *A. pullulans* can inhibit *E. lata* according to our study in commercial vineyards and *in vitro* when cultured together with *E. lata*, but *A. pullulans* showed less or no inhibitory effects against *E. lata* within the *in planta* experiments. More research is required to further

understand the potential protective effect of *A. pullulans* in grapevines. Future research opportunities are outlined in sections 5.4 in this chapter.

5.3.1 Two *A. pullulans* strains showed unique effects

All *A. pullulans* strains had significant inhibitory effects on *E. lata* when dual-cultured on PDA plates, but only *A. pullulans* 3057 and 21143 increased leaf presence with co-inoculation of *E. lata* than canes inoculated with *E. lata* alone. None of the four strains increased leaf or root presence or reduced stain size in canes. Canes showed the highest average stain size when inoculated with two PDA plugs, this made our evaluation of fungal antagonism difficult. Also, this might be an explanation of why the results of *in planta* test did not match those *in vitro* or *in silico*. *A. pullulans* 21143 also showed outstanding results *in planta*: canes inoculated with *A. pullulans* 21143 showed lowest stain size among all *A. pullulans* treated canes, and lower than canes treated with *E. lata* or PDA only. However, *A. pullulans* 21143 was also the most inhibited *A. pullulans* strain by *E. lata* in dual-inoculation tests.

5.3.2 Potential mechanisms related to our research

In Chapters 3 and 4 both methods applied involved culturing fungi, i.e. in either pure cultures on media (Chapter 3) or grapevine canes (Chapter 4), These provided two environments in which *A. pullulans* and *E. lata* could have direct interaction and reveal how they might both contribute to affect grapevine health.

A positive *E. lata* inhibition result *in vitro* does not necessarily indicate protection effects in plants of the extra organism present, the plant. This tripartite engagement results in a complicated interaction of each fungus with the other and with plant tissues. Apart from known suppression effects (for instance, competition for basic space and nutrients, toxic compounds secretion and activation of the plant defense system), fungi can express different growth status in plates and plants significantly (Kavanagh 2017). That is why we need to apply both *in vitro* and *in planta* tests to demonstrate if *A. pullulans* would protect grapevines from *E. lata* infection and if it does, the mechanism. Positive suppression in

plates normally suggests *A. pullulans* can actively compete for resources or (and) produce toxic compounds against *E. lata*. We hoped co-inoculation of two species in canes can result in smaller stain size or reduced *E. lata* presence than canes only inoculated with *E. lata in planta*. If it happened, we would be confident that *A. pullulans* can directly interfere with *E. lata* development in canes or indirectly inhibited *E. lata* by triggering plant defense responses. However, if we took stained cane as an indicator of "sick plant", we might conclude that *A. pullulans* is the plant pathogen. But it was more probably that our project needs to be improved.

Although the circumstance our study is promising, further detailed research is required *in planta* to detect an inhibitory impact in that environment. Furthermore, very detailed research is required to demonstrate the mechanism behind any protective effects that *A. pullulans* might show in grapevines after inoculation.

We found surprising results in this study: *E. lata* may have promoted *A. pullulans* development in canes. Co-inoculation of *E. lata* and *A. pullulans* resulted in higher *A. pullulans* presence than canes treated with *A. pullulans* itself (section 4.4.3.3). *E. lata* and other fungal phytopathogens can synthesize cellulase to degrade plant tissue and obtain nutrients from starch and cellulose (Octave, Roblin et al. 2008). Thus, glucose degraded from the cane or added PDA cultures can provide extra nutrients to nearby micro-organisms within plants. This may explain why we noticed canes showed high average stain size (3.33 cm) in PDA-only treated canes. In this way, stain size might be a wrong indicator of health status. Otherwise, we may have to conclude that *E. lata* can protect grapevines from other inner micro-organisms.

In the *in planta* experiments (Chapter 4), no strain of *A. pullulans* reduced the incidence of *E. lata* and *A. pullulans* 20345 was the only strain that decreased the cane stain size when co-inoculated with *E. lata*, compared to *E. lata*-only inoculated. These results indicated two potential theories: 1. *A. pullulans* may not protect grapevine from the infection of *E. lata*; 2. to choose cane stain length sizes as indicators of GTD symptoms was a poor indicator of the inhibition of the pathogen or 'health'. Alternatively, the experiment ran for insufficient time (83 dpi) or the positioning of plants within the shade house resulted in erroneous outcomes (section 4.4.5)

5.3.2.1 Plant defense system

Plant defense system is a key strategy involved in plant-micro-organism interaction (Mehdy 1994). It is the predominant suppression method in response to pathogen and also one major pathway of *A. pullulans* protective effect. Once entered into plants, phytopathogens generate effectors as weapons to recognize and attack plant cells related to defense response (Jones and Dangl 2006). Plants do not have mobile immune cells in animals and their defense responses rely on two pathways: innate immunity of each call and systemic signals from infection sites (Dangl and Jones 2001). Combination of these two levels of immune responses contributes to inhibited disease infection. *E. lata* can trigger plant defense gene expression in grapevine cell suspension cultures after 48 hours (Mutawila, Stander et al. 2017). In addition to typical plant defense responses, grapevines can also block further colonisation of *E. lata* (and other wood pathogens) by producing gums and tyloses in their xylem vessel (Mutawila, Stander et al. 2017). Often the blocking of plant vessel is the cause of foliar symptoms (as mentioned in chapter 4.4.2).

Molecular detection uncovered strange results: The co-inoculated *E. lata* has in fact increased presence of *A. pullulans* than canes treated with *A. pullulans* alone. Therefore a new hypothesis raised up: *E. lata* has promoted *A. pullulans* growth by either degrading plant tissues or suppressing plant defense system. Since *A. pullulans* can trigger plant defense responses in grapevines (and thus protect plants against pathogens), it is also possible that grapevines are restricting *A. pullulans* growth by defense responses. The compounds secreted by *E. lata* to suppress plant immune response may also ease plant stress on *A. pullulans* (Mauro, Vaillant et al. 1988). There are not sufficient studies about how *A. pullulans* was inhibited by plant defense system.

Despite *A. pullulans* has been applied to be an effective biocontrol agent to *B. cinerea* and *Geotrichum citri-aurantii* (causal agent of grapevine sour rot disease) in grapevines and other crops, we are still unsure about mechanisms behind these positive impacts and a significant reduction of average stain size seems reasonable. Thus though it would have been an experiment for us to “prove” this hypothesis instead of exploring a new frontier.

Grape trunk glucose weight decreases significantly when infected by *E. lata* and this indicates a change of chemical compounds caused by *E. lata*(Rolshausen, Greve et al. 2008). Such variation of plant tissues may increase the accessibility of nutrients for other fungi which have lower wood degrading enzyme activities. And this approach may even appear in multiple fungal pathogens, resulting in a positive cycle for fungal disease.

Grapevines have their own defense system in responding to pathogen infection, often through secreting phytoalexins such as resveratrol and δ- viniferin (Schubert, Fischer et al. 1997). These chemicals can inhibit pathogen growth and reduces infectious stress, but some pathogens have also developed ways to bypass plant defense response. *B. cinerea* and other pathogens cause Botryosphaeria die-back. They can metabolize the phytoalexins and avoid being significantly suppressed by resveratrol or viniferin (Stempien, Goddard et al. 2017). There are some other endophytic micro-organisms showing protective effect: *Pseudomonas* sp. Strain PsJN showed inhibitory effects on *B. cinerea* (Barka, Gognies et al. 2002).

A. pullulans showed inhibitory effect against both fungal and bacterial phytopathogens and there are multiple mechanisms behind this phenomenon (Ippolito, El Ghaouth et al. 2000, Di Francesco, Roberti et al. 2015, Pinto, Custodio et al. 2018, Don, Schmidtke et al. 2019). One interesting point of view is: Does *A. pullulans* inhibit bacteria and fungi in different ways? Since bacteria and fungi are both morphologically and physiologically different, triggered pathways may also very distinct. There are not enough studies in this field so related evaluation will uncover more opportunities to apply *A. pullulans* in more crops.

Apart from direct interaction among fungi within plants, the triggered plant defense system can restrict the invasion and growth of fungal pathogens (Gadoury, CADLE‐DAVIDSON et al. 2012). Plants have developed a number of defense strategies towards fungal pathogens. To inhibit biotrophic fungi such as *Erysiphe necator* (causal agent of mildew powdery in grapevine), two major plant defense pathways exist, i.e. penetration resistance and programmed cell death. These either modify the cell wall structures and prevent the formation of haustorium to engender penetration resistance, or induce the death of invaded cell resulting in programmed cell death. In addition, the innate immune response in plant cells works as the first line of defense response. It provides sufficient protection against most biotrophic infections (Qiu, Feechan et al. 2015). Apart from directly induced a

plant defense system, a pathogen-triggered biosynthesis of secondary metabolites are a well-studied plant response to pathogens and this plays an important role in plant immunity (Bednarek and Osbourn 2009).

In some cases, grapevines may not show any symptoms of *E. lata* or *E. lata* DNA cannot be detected even after inoculation (Halleen, Fourie et al. 2016). Unsuccessful pathogen occupation and disease development may possibly be due to effective plant defense system (see section 5.3.2 and this section) or a result of a stable and resilient microbiome that may contain BCAs such as *A. pullulans* that act either by niche occupation or by active inhibitory effects (Pinto, Custodio et al. 2018). Similarly, the presence of cane discolouration does not necessarily correlate with the presence of a pathogen within the symptomatic tissues (section4.4 2.2). The plant may have already cured the infection in this specific area and the may be evidence of previous defense response.

5.4 Future Directions

5.4.1 Apply other strains of *A. pullulans* and try different treatments

A. pullulans has attracted interest in controlling *Pseudomonas syringae* pv. *actinidiae* (Psa) in kiwifruit for decades (McCormack, Wildman et al. 1995). And some commercial products are using *A. pullulans* as major active ingredients (e.g. Aureo Gold™, Arysta LifeScience, New Zealand). A combination of *A. pullulans* with plant defence elicitor showed a better reduction of foliar symptoms in kiwifruit (de Jong, Reglinski et al. 2019). In the study of de Jong, *A .pullulans* CG 163 triggered an enhanced expression of plant defense system at 24 h post inoculation, and the combination of both *A. pullulans* CG 163 and plant defence elicitor showed the strongest effect among groups. Our *A. pullulans* strain are different from their isolate (CG163) or commercial Aureo Gold™ (YBCA5). It is unknown to what extent these strains are different, but it would be beneficial if we can try their strains in future to test impact on grapevines.

5.4.2 Change *in planta* treatment methods

Another suggestion is to spray pure fungal solutions on leaves or roots, instead of inoculating with plugs into each cane particularly still attached to nutrient-rich medium PDA. There are two principal reasons for this. Inoculation of PDA would add extra nutrients to fungi in canes, including *A. pullulans*, *E. lata* and fungal species already colonized in plant tissues. Thus fungi would grow too fast and the plant has insufficient time to respond. Not many studies inoculate PDA directly into plant tissues, instead, they use water agar to avoid extra nutrients (Naqqash, Hameed et al. 2016). The other reason is spraying fungal solutions can mimic the natural entrance of *A. pullulans* into plants. Naturally, it is unlikely to have a large number of fungi (not only for *A. pullulans* and *E. lata*) entering into the wound at once. Fungal phytopathogens enter into plants by either spreading spores with rain, water or transmitted by insects (Reddy 2016). They would allow some time for fungi to develop and colonize within plants (Bethlenfalvay, Pacovsky et al. 1982). It might be helpful if we can treat plants with *A. pullulans* first and wait for a few weeks to allow *A. pullulans* colonize plant tissues. Hence apart from inducing plant defense system, early occupation of inner space can enhance potential protective effects. In addition, some non-inoculated canes can

be appropriate negative controls within *in plant* tests. For these canes, they should be both un-drilled and drilled but non-inoculated to eliminate the effect of drilling and medium.

5.4.3 Quantify amount of inoculated fungal isolates

The inoculated amount of fungal was unknown, though we managed to transfer mycelial plugs with even size. *A. pullulans* is a yeast-like fungus so there was no tight connection among cells (Gostinčar, Turk et al. 2019). By contrast, *E. lata* mycelial makes the predominant structure for colonization (Živković, Vasić et al. 2019). So the density of inoculated fungal amount at the start of inoculation is unknown. In Chapter 2, it was observed that *A. pullulans* has weak protection against *E. lata* in vineyards using historical data; thus it is possible a larger amount of *A. pullulans* than *E. lata* could express positive results.

5.4.4 Add herbicide in dual-culture tests

Culturing *A. pullulans* and *E. lata* strains *in vitro* with additional herbicide in medium would be a promising experiment in future. As discussed in Chapter 3 Section 3.4.5, added glyphosate would mimic growing conditions seen in the contemporary management compared with future managed conditions. Glyphosate may influence the metabolites of *A. pullulans* and *E. lata*, and thus reveal different *in vitro* results.

5.4.5 Prolong project period

In planta tests could be enhanced with a longer study. As stated, stain size, presence of leaf and root may not represent grapevine health status/GTD symptoms. An alternative method that might be more representative of natural field plants would be to grow inoculated canes into individual plants or perform inoculations into young and GTD-free grapevine trunk. Due to the project time limitation, our indicators of GTD symptoms cannot comprehensively reflect real fungal infection within plants. If we can monitor changes of treated canes (or

trunks) over years in an enhanced experiment, we will be able to analysis *A. pullulans* effect on GTD at a better experimental scale.

5.4.6 Sequence trunk/cane tissue before inoculation

One appropriate improvement of the current study is to evaluate existing fungal OTUs prior to inoculation. We cannot guarantee selected canes are fungi-free, even multiple approaches were performed to minimise potential fungal exists (select young canes, cut from healthy plants and sterilize equipment to reduce contamination). In Chapter 4 Section 4.3.4, real-time PCR showed our canes had low possibility to have *E. lata* before inoculation. But they may still contain other micro-organisms we did not test. These fungi or bacteria might be causing infection in PDA-inoculated canes. Evaluate contained species would be an important work for other researchers in the future.

5.5 Summary

This project characterised the potential protective effect of *A. pullulans* on *E. lata in silico, in vitro* and *in planta*. *A. pullulans* has reduced the abundance of *E. lata* in NGS data and inhibited colony radii of *E. lata* on PDA plates. This protective effect did not appear in inoculated grapevine canes, in other words, co-inoculation of *A. pullulans* cannot significantly reduce stain size or *E. lata* presence. Also, canes treated with two PDA plugs expressed the highest average stain size among treatments. This in-cooperated of *in planta* results suggested selected *A. pullulans* strains had no statistical effect inhibiting the development of *E. lata*. It is also possible we chose incorrect methods to evaluate plant health status.

A. pullulans is still a potential candidate for biocontrol of *E. lata* and related GTDs. One suggestion of this research is that the *A. pullulans* strains can be further evaluated in terms of its ability to prevent the development of GTD symptoms with representative study objects. Also we need to alter *in planta* study methods to better mimic growing conditions in vineyards.

Conversely, a negative result (*A. pullulans* did not inhibit the growth of *E. lata*) in plates could also have less severe or disappeared GTD symptoms when *A. pullulans* and *E. lata* were both inoculated into vines. Under this circumstance, the likelihood of plant defense as a major protective factor rises. However, we could not uncover whether this plant defense effect was induced by *A. pullulans* or simply *E. lata* if all of these inoculated (include control groups and test groups) expressed no *E. lata* infection.

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Appendix

1. Protocols for fungal DNA extraction

1.1 Fungal DNA extraction by DNeasy Plant Mini Kit (QIAGEN)

Add ethanol to Buffer AW1 and Buffer AW2 concentrates, preheat a water bath at 65°C. Then disrupt samples in tubes with spherule and ground it mechanically. Add 400 µL Buffer AP1 and 4 µL RNase A, vortex the mixture and incubate for 10 min at 65 °C, invert the tube 3 times during incubation. Then add 130 µL Buffer P3, mix and incubate tube for 5 min on ice. Centrifuge the lysate for 5 min at 20,000 x g, pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube and centrifuge for 2 min at 20,000 x g again. Transfer the flow-through into a new tube without disturbing the pellet and add 1.5 volumes of Buffer AW1, and mix by pipetting. Transfer 650 µL of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuge for 1 min at 6000 x g, discard the flow-through and repeat it again. Place the spin column into a new 2 ml collection tube, add 500 µL Buffer AW2 and centrifuge for 1 min at 6000 x g, then discard the flow-through. Add another 500 µL Buffer AW2 and centrifuge for 2 min at 20,000 x g. Transfer the spin column to a new 2 ml micro centrifuge tube, add 100 µL Buffer AE for elution, incubate for 5 min at room temperature, centrifuge for 1 min at 6000 x g and repeat this step.

1.2 Fungal DNA extraction protocols of CTAB methods:

1. Wood tissues were homogenised in 800 µL CTAB buffer with 2.3-mm steel beads for 90 seconds.

2. Homogenised samples were incubated at 65 °C for 90 minutes.

3. Use equal volume of chloroform: isoamyl alcohol (24:1). Centrifuge once again at 15,000 x g for 15 minutes.

4. Precipitate DNA solution with isopropanol (0.54 volume) and 7.5 M ammonium acetate (0.08 volume), incubate at -20°C overnight. Centrifuge at 15,000 x g for 30 seconds.

5. Discard the supernatant, rinse sample with 70% ethanol then dry in air for 20 minitues.

6. Resuspend DNA pellet in 40 µL of super pure water, then incubate at 37 °C for 30 miniutes.

After extraction DNA from cane tissues, we processed PCR for fungal and bacterial DNA amplification.

2. GTD symptoms recording in vineyards and NGS protocols in Chapter 2

2.1 GTD symptoms recording

The symptoms and NGS data in this study are based on recording in year 2017-2018. Vineyard blocks in Marlborough and Hawke's Bay were assessed for visual symptoms of GTDs in the 2016/2017 season and 2017/2018 season. In each vineyard, 816 to 2508 vines were surveyed and the same vines were surveyed for two seasons. The GTD symptoms were recorded (Figure A2.1) as canker (C), half head (H), stunted shots (S), retrunk (R), young (Y), gap (G) and dead (D). Trunk symptoms and foliar symptoms were detected visually by walking along each bay and inspecting the growing status of vines. Although it is more accurate to detect GTD by cutting trunks vertically and observing cankers or necrosis within the trunk, this destructive method is not practical.

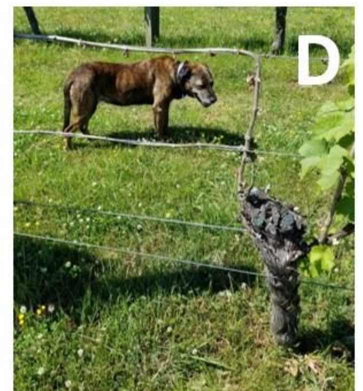


Figure A2.1. GTD symptoms recorded in selected vineyards at Marlborough and Hawke's Bay for VE programmes in November 2018. A: Canker on arm caused death and stunted shoots, leaves were missing under yellow arrow; B: Infection of GTD started in the circle and affect arm on both sides. Reduced water and nutrients with phytotoxins produced by pathogens significantly inhibit the growth of shoots and leaves; C: Retrunk Infected trunk was removed and new trunk was planted from the uninfected main trunk; D: A typical symptom of entirely dead arm.

2.2 Preparation of samples to identify fungal infection and OTU in grapevines

For the fungal identification, samples were collected from Sauvignon blanc and Pinot noir vines in vineyards at Marlborough and Hawkes' Bay.

Knives were used to remove bark tissues and then the exposed trunk was drilled with sterilized 4-mm drill bits to collect a single cane sample each year. In order to minimise contamination, drill bits were changed between each vine and used bits were sterilised in 3% (v/v) hypochlorite solution. After sampling, wounds in cane were sealed by linseed wood putty to reduce infection risk. Collected tissues were placed in 4 ml cryogenic tubes and stored in liquid nitrogen at -80°C.

Tissue samples were homogenized using an eight-well bead beater. Half of the total DNA samples collected from each five-vine composite were mixed up for NGS, and the rest samples were stored in liquid nitrogen for future assessment.

2.3 Fungal DNA extraction by CTAB methods

Grapevine tissues often contain a large amount of secondary metabolites and this makes DNA extraction and purification difficult (Mundy, Vanga et al. 2018). It is essential to manage a timely protocol, to avoid degradation of DNA or contaminated by environmental DNA. DNA extractions were undertaken by cetyl trimethylammonium bromide (CTAB) method, procedures have subtle modifications (Mundy, Vanga et al. 2018) and the same as the Appendix 1.2.

2.4 Amplification and NGS for fungal ITS region

To identify the fungi within each vine DNA metabarcoding was performed targeting the amplified fungal ribosomal internal transcribed spacer 1 (ITS1) gene. Primers for fungal ITS amplification include the forward primer NSI1a and reverse primer 58A2R (Leski, Gregory et al. 2010):

NSI1a: 5'-GATTGAATGGCTTAGTGAGK-3'

58A2R: 5'-AGTCCTGCGTTCTTCATCGAT-3'

PCRs were carried out in 20 µL volume in total each well (Table A2.1). Amplification cycles were: Denaturation at 94°C for4 min, followed by 35 cycles of 94°C for 20 s, annealing at 50°C for 20 s, extension at 72°C for 60 s and a final extension at 72°C for 7 mins.

Table A2.1. Content of each well for fungal PCR of ITS1 region, modified from Dion Mundy (Mundy, Vanga et al. 2018). Each reaction tube contained 20 µL of total reagents.

Content	Volume
Template sample DNA	1 µL
KAPA3G plant DNA polymerase	0.2 U
PCR master mix-KAPA3G plant PCR kit	10 µL
58A2R 10 µM	0.5 µL
NSI1a 10 µM	0.5 µL
	In total 20 µL

In addition to performing the PCR with ITS1 primers (NSI1a and 58A2R), a PCR was also conducted to amplify the ITS2 region of fungal DNA using 36 tissue samples with ITS2 primers (ITS3F: 5'-GCATCGATGAAGAACGCAGC-3' and ITS4R: 5'-TCCTCCGCTTATTGATATGC-3'), same PCR protocols were used as PCR for ITS1.

Samples were sent to Massey Genome Service (Massey University, Palmerston North) for sequencing. NGS was performed using the Illumina MiSeq platform to generate 300 bp paired–end reads (http://www.illumina.com/systems/miseq/applications.html). Results came back with fungal OTUs in each locus.

In each VE site, vines were assessed by visual observation for GTD symptoms and NGS for the fungal microbiome. Researchers of Research Aim 1.3 have done experiments at Blenheim, Marlborough. This NGS analyses results indicated the existence of common fungal species and viruses that cause trunk diseases of grapevines and viral species associated with disease (Table A2.1). This pathogen map provided us with crucial information about what pathogens were affecting vines and co-existence of potential BCAs, such as *A. pullulans*.

3. Statistical analysis of Chapter 2

Information was provided by Beatrix Jones with minor modifications for wording and paragraph. Due to the limitation of thesis length, some programming contents are not shown in the appendix.

3.1 GTD symptoms in monitored vineyards

During the 2017/2018 season in Marlborough, the incidence of visual symptoms ranged from 2.4% to 18.9% of vines monitored. Compared with previous seasons, for five of the twelve sites in Marlborough, 2017/2018 had the highest percentage of vines with symptoms (Figure A3.1). For three of the sites, the 2017/2018 season had a lower percentage total symptoms than previously observed.

In Hawke's Bay, seven sites recorded with the highest percentages of symptoms in the 2017/2018 season and two the lowest percentage of symptoms in the 2017/2018 season and two the lowest percentage.

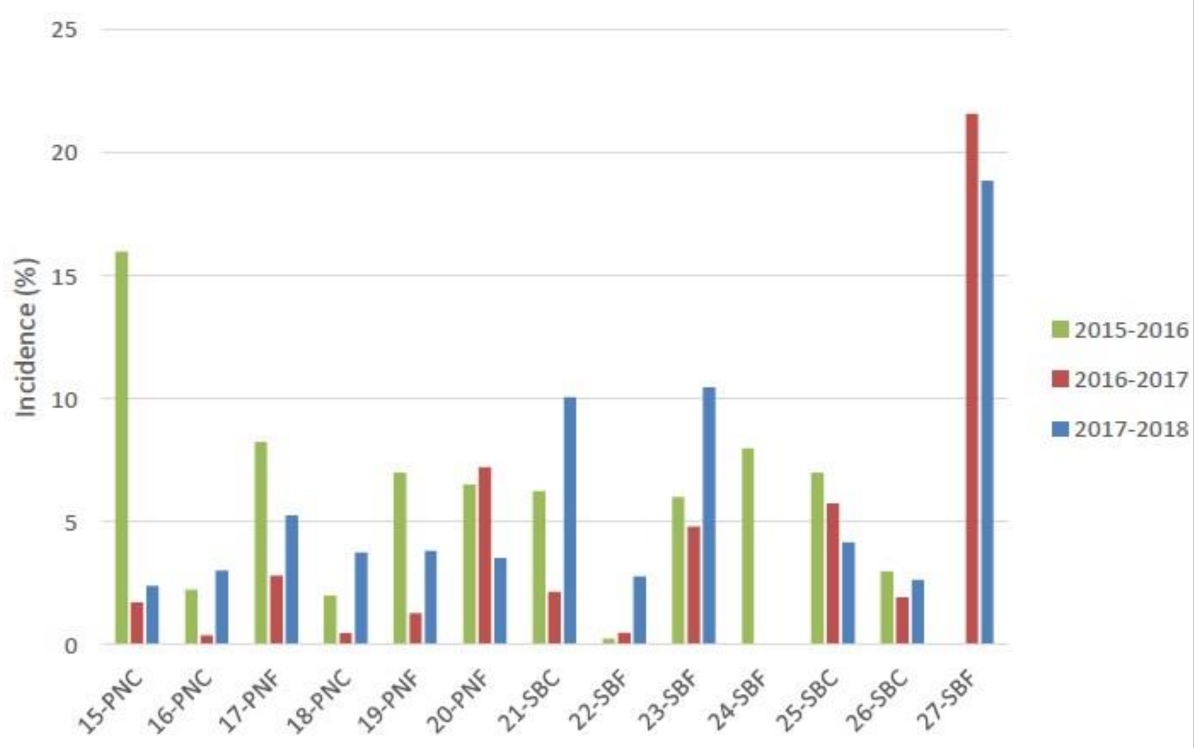


Figure A3.1. GTD visual symptoms recorded from season 2015/2016 to season 2017/2018 at VE sites in Marlborough (Mundy, MacDiarmid et al. 2018). GTD symptoms are recorded as mentioned in last sections: stunted shoots, external cankers and missing spur positions. Site 24 SBF was removed after the first season and replaced by 27 SBF. Data in 2015/2016

was collected from 200 random vines. Data is summarized from an internal report of VE system.

3.2 Data analysis 1 of combined *A. pullulans* and *E. lata* levels with variables in vineyards (11 pages).

Section 3.2 and section 3.3 were original letters from Beatrix Jones.

Analysis of *A. pullulans* and *E. lata*, 2 years data

Beatrix Jones

OTU counts have been read in and aggregated by species name. Samples with less than 1000 total reads have been excluded. Counts have been divided by the total reads in each sample to create proportions.

We want a linear mixed model describing the effect of variety, management, and year on *A. pullulans* and *E. lata*, controlling for vineyard effects. We are also interested in any association between them controlling for vineyard and any other significant effects.

We would also like a model that predicts symptoms based on these and their interaction, controlling for vineyard effects.

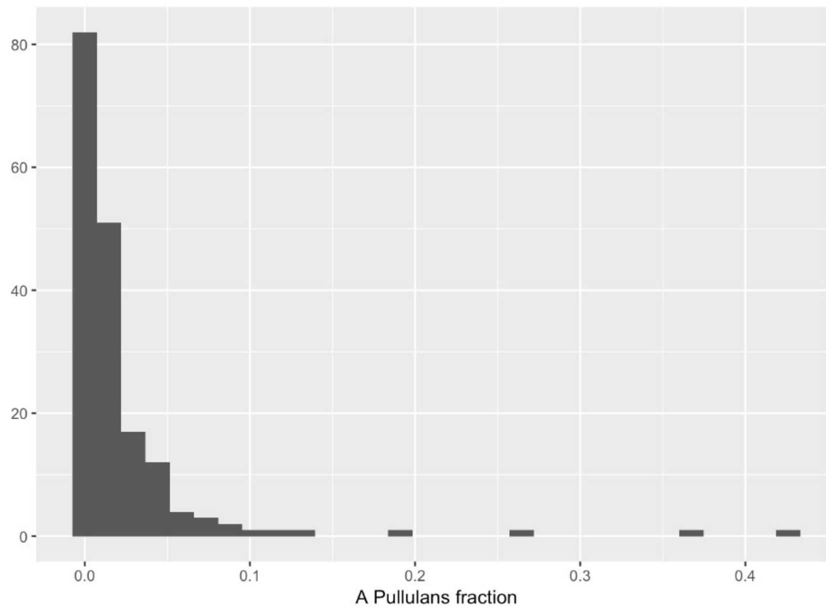
Transformations

First, we consider whether transformation of the *A. pullulans* and *E. lata* counts are necessary, and what the transform might be.

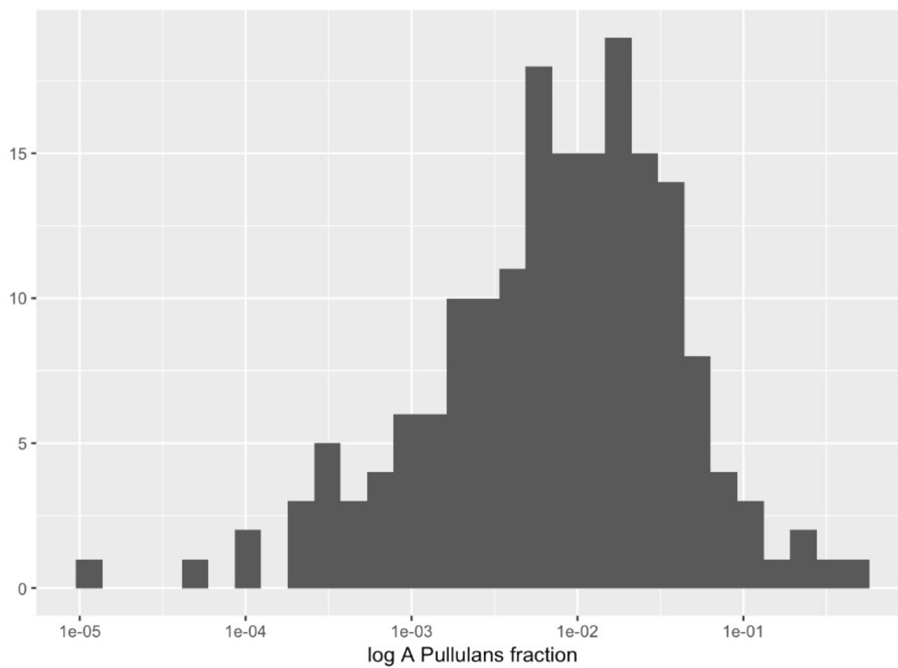
A. pullulans:

Question: Does the *A pullulans* need to be transformed? Is the (natural) log transform suitable?

```
## `stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```




```
## `stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```



Conclusion: Transformation needed. A (natural) log transform appears appropriate.

Question: Does Variety and or Management and or Year affect *A. pullulans*, treating Vineyard as a random effect?

We fit a series of nested models to the transformed fraction of *A. pullulans* and compare them with the likelihood ratio tests. We check the residuals of the chosen model.

```
names(loci3)[4]<-"Vineyard2"
Vineyard<-substr(loci3$Vineyard2,1,2)
modAP.full<-lmer(AP~(1|Vineyard)+Management+VE.Year+Variety,
data=loci3)
modAP.noyear<-lmer(AP~(1|Vineyard)+Management+Variety,
data=loci3)
anova(modAP.noyear, modAP.full)
```

```
## refitting model(s) with ML (instead of REML)
```

```
## Data: loci3
## Models:
## modAP.noyear: AP ~ (1 | Vineyard) + Management + Variety
## modAP.full: AP ~ (1 | Vineyard) + Management + VE.Year + Variety
##              Df    AIC    BIC  logLik deviance Chisq Chi Df
Pr(>Chisq)
## modAP.noyear  5 654.21 670.12 -322.10   644.21
## modAP.full    6 656.14 675.23 -322.07   644.14 0.067      1
0.7958
```

```
## refitting model(s) with ML (instead of REML)
```

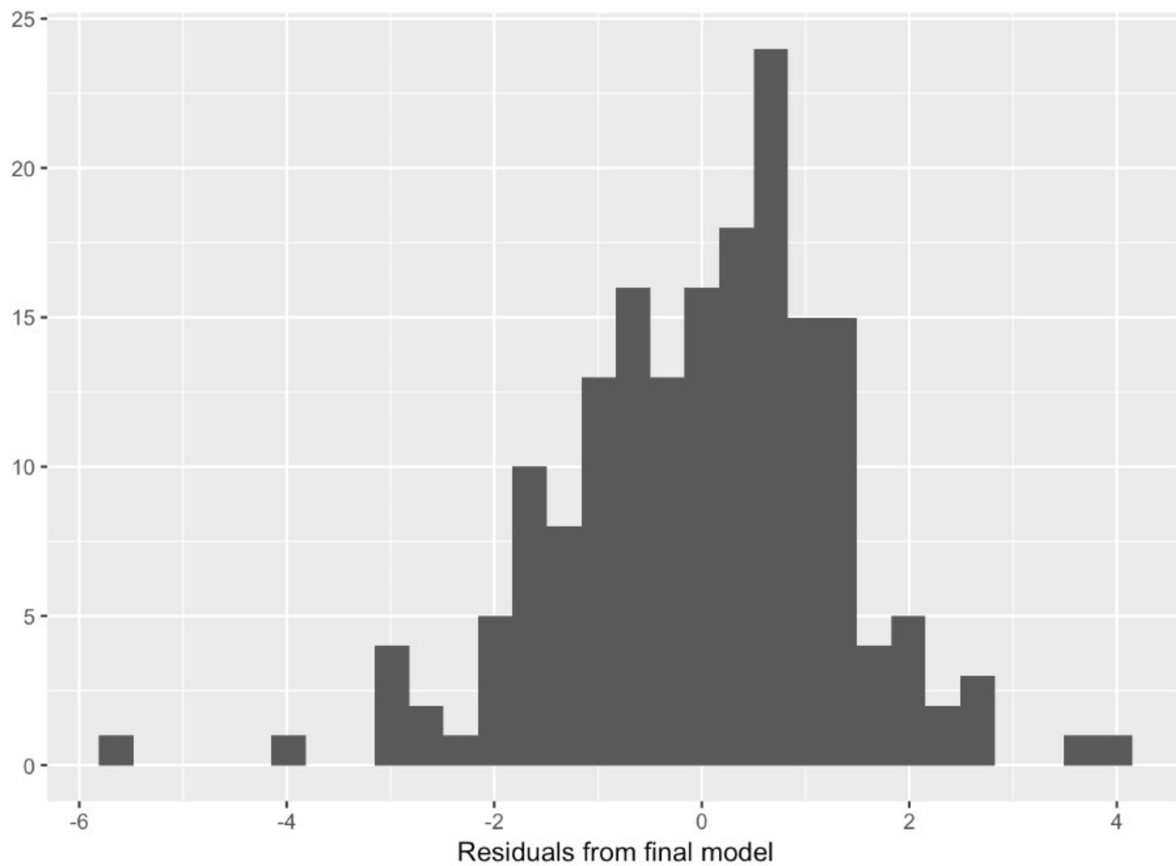
```
## Data: loci3
## Models:
## modAP.noVar: AP ~ (1 | Vineyard) + Management
## modAP.noyear: AP ~ (1 | Vineyard) + Management + Variety
##              Df    AIC    BIC  logLik deviance  Chisq Chi Df
Pr(>Chisq)
## modAP.noVar   4 658.43 671.16 -325.22   650.43
## modAP.noyear  5 654.21 670.12 -322.10   644.21 6.2248      1
0.0126 *
```

```
summary(modAP.noyear)
```

```
## Linear mixed model fit by REML ['lmerMod']
## Formula: AP ~ (1 | Vineyard) + Management + Variety
##    Data: loci3
##
## REML criterion at convergence: 644.5
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -4.0230 -0.5954  0.1014  0.6134  2.8739
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Vineyard (Intercept) 0.8344   0.9134
##  Residual             1.9486   1.3959
## Number of obs: 178, groups:  Vineyard, 12
##
## Fixed effects:
##             Estimate Std. Error t value
## (Intercept)  -3.6228     0.4330  -8.367
## ManagementF  -1.5210     0.4619  -3.293
## VarietySB    -1.1981     0.4845  -2.473
##
## Correlation of Fixed Effects:
##             (Intr) MngmnF
## ManagementF -0.502
## VarietySB   -0.524 -0.071
```

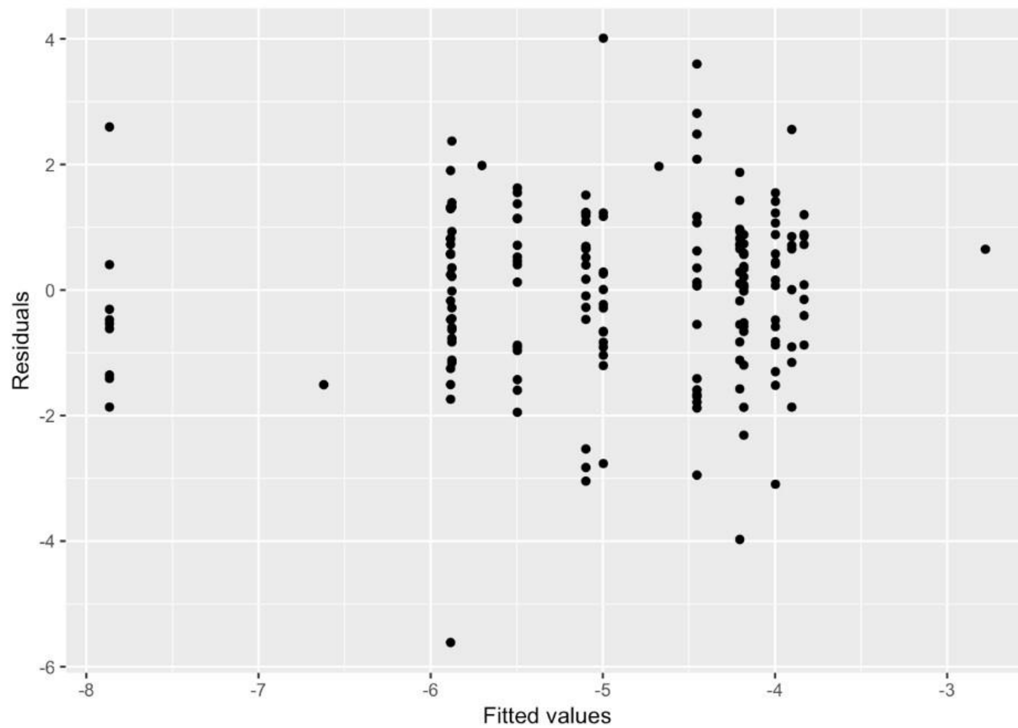
```
par(mfrow=c(1,2))
qplot(residuals(modAP.noyear), xlab="Residuals from final model")
```

```
## `stat_bin()` using `bins = 30`. Pick better value with `binwidth`.
```




```
qplot(fitted(modAP.noyear), residuals(modAP.noyear), xlab="Fitted values", ylab="Residuals")
```

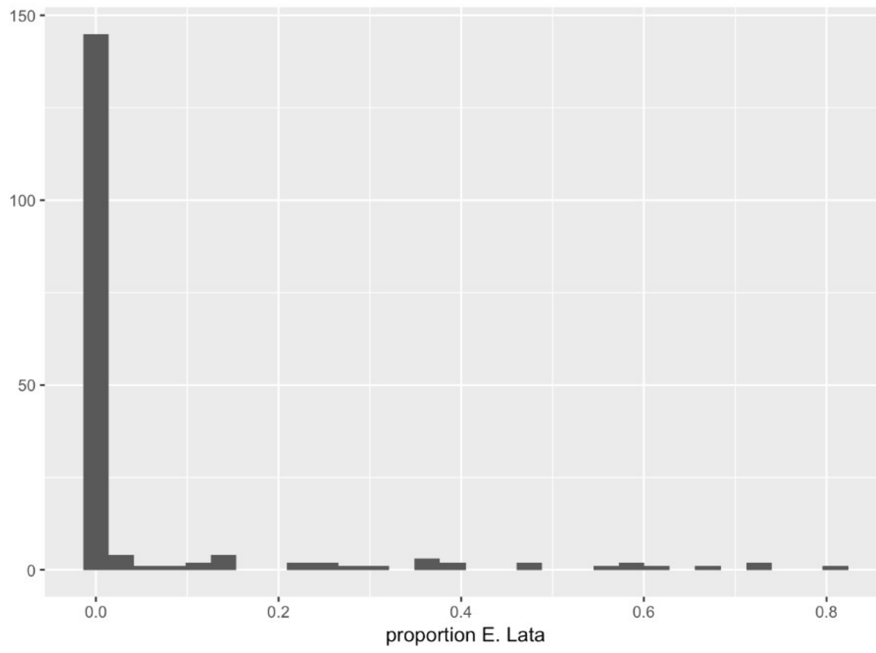
Conclusion: Year **does not** appear to affect the level of *A. pullulans*, but Management and Variety both do, with future Management resulting in less *A. pullulans*, and Sauvignon Blanc vineyards having less *A. pullulans*. There is some lack of fit at the lower end of the model.

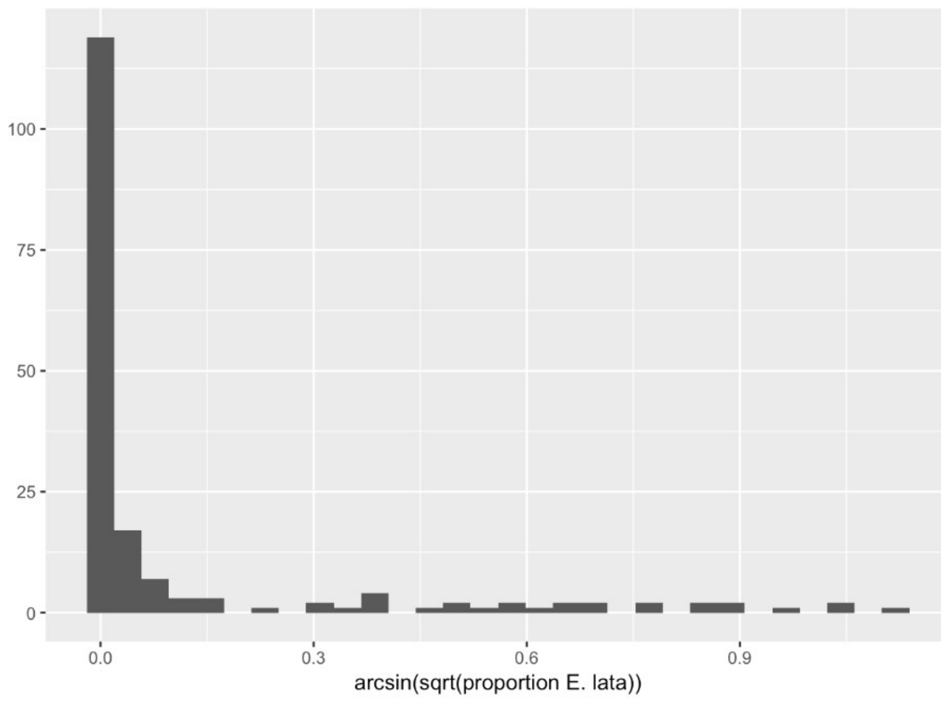


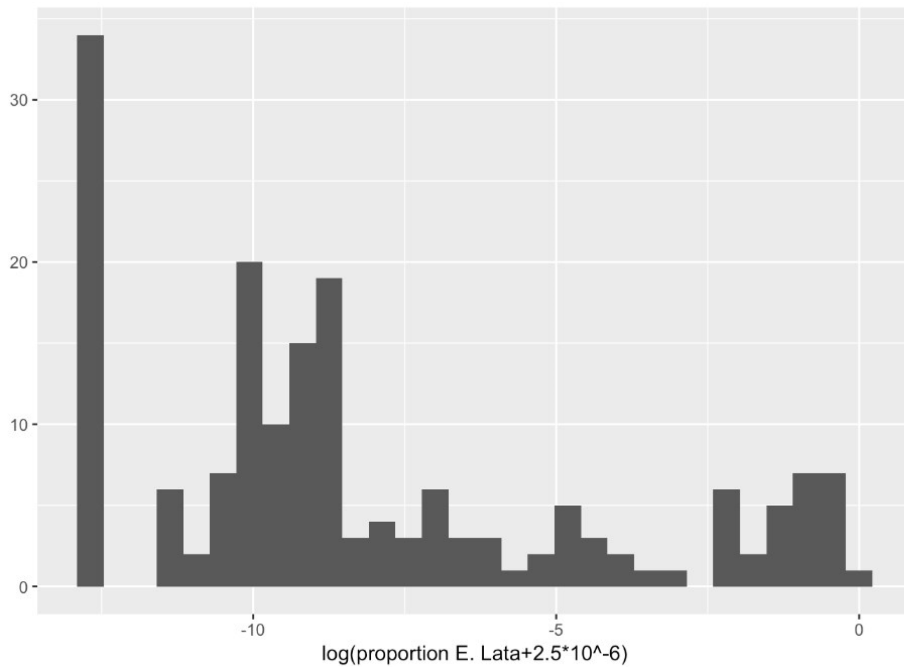
E. lata

Transformation of *E. lata* is more challenging because there are some zero values. We will try the log transform after adding half the minimum, non-zero proportion, over all species. The arcsin-sqrt transform was also considered.

Question: Does the *E lata* need to be transformed? Is the arcsin-square root transformation suitable? Or is the log transform, (after addition of half the minimum, non-zero proportion, over all species to cope with zero values) better?

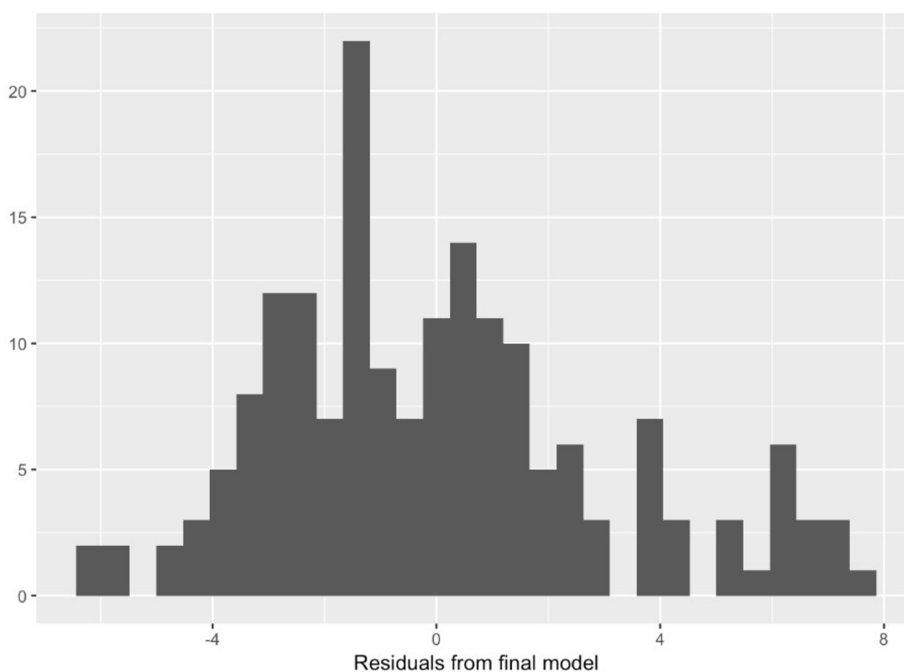

150
100
50
0
0.0
0.2
0.4
0.6
0.8
proportion E. Lata

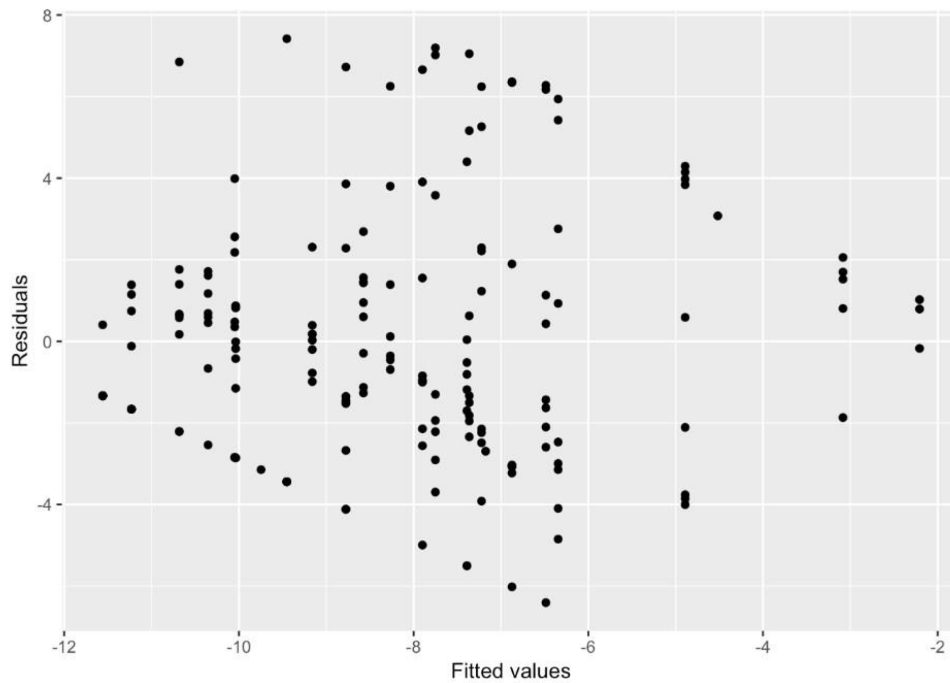

100
75
50
25
0
0.0
0.3
0.6
0.9
arcsin(sqrt(proportion E. lata))



The **log transform** appears better. Unfortunately subsequent analyses have some sensitivity to the constant chosen to boost the zero values. We will highlight this where relevant.

Question: Is *E. lata* affected by Management, Year, or Variety, while accounting for vineyard effects?





Conclusion *E. lata* is not affected by Management. There is week evidence ($p < 0.1$) that it is affected by year, with Year 2 having less *E. lata*, and is affected by Variety, with Sauvignon Blanc vineyards having more *E lata*. Assumptions of the linear model are imperfectly satisfied.

Question Is *E. lata* associated with *A. pullulans*, after controlling for vineyard, Year, Management, and Variety?

```
## Linear mixed model fit by REML ['lmerMod']
## Formula: EL ~ (1 | Vineyard) + Variety + VE.Year + Management + AP
##    Data: loci3
##
## REML criterion at convergence: 921.3
##
## Scaled residuals:
##      Min       1Q   Median       3Q      Max
## -2.32707 -0.68757 -0.07082  0.56406  2.45036
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Vineyard (Intercept) 7.578    2.753
##  Residual             9.203    3.034
## Number of obs: 178, groups:  Vineyard, 12
##
## Fixed effects:
##              Estimate Std. Error t value
## (Intercept) -11.23280    1.32330  -8.488
## VarietySB     2.26435    1.27595   1.775
## VE.YearY2    -0.86713    0.47956  -1.808
## ManagementF  -0.08515    1.20831  -0.070
## AP           -0.52891    0.16645  -3.178
##
## Correlation of Fixed Effects:
##             (Intr) VrtySB VE.YY2 MngmnF
## VarietySB   -0.349
## VE.YearY2   -0.155 -0.033
## ManagementF -0.271 -0.077 -0.053
## AP           0.452  0.155 -0.027  0.229
```

Conclusion There is evidence of an association after controlling for Vineyard, Variety, Management, and Year, with *A pullulans* reducing the level of *E. lata*.

Sensitivity of existing model

Question is the model sensitive to the constant used in the transformation of *E. lata*?

We try two alternatives: 1.26x10^-6 (half the current value), and 4.1 x 10^-6, half the minimum *E lata* measurement.

```
## Data: loci3
## Models:
## modEL.noAP.sen2: EL.sen2 ~ (1 | Vineyard) + Variety + Management +
VE.Year
## modEL.AP.sen2: EL.sen2 ~ (1 | Vineyard) + Variety + Management +
VE.Year + AP
##                  Df    AIC    BIC  logLik deviance Chisq Chi Df
Pr(>Chisq)
## modEL.noAP.sen2  6 937.01 956.10 -462.50   925.01
## modEL.AP.sen2    7 928.72 950.99 -457.36   914.72 10.29      1
0.001337
##
## modEL.noAP.sen2
## modEL.AP.sen2   **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Conclusion: There is still strong evidence of a negative association between *E. lata* and *A. pullulans*, regardless of the choice of constant.

Question: What happens if we model presence/absence of *E. lata* via a generalized linear mixed model, to avoid use of the constant?

```
## Data: loci3
## Models:
## gmodEL: ELpres ~ (1 | Vineyard) + Management + Variety + VE.Year +
Management
## gmodEL.AP: ELpres ~ (1 | Vineyard) + Management + Variety + VE.Year
+ Management +
## gmodEL.AP:     AP
##            Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
## gmodEL      5 157.31 173.22 -73.657   147.31
## gmodEL.AP   6 159.31 178.40 -73.655   147.31 0.0038      1      0.951
```

Conclusion: *A. pullulans* is not helpful in predicting the presence/absence of *E. lata*.

Symptom models

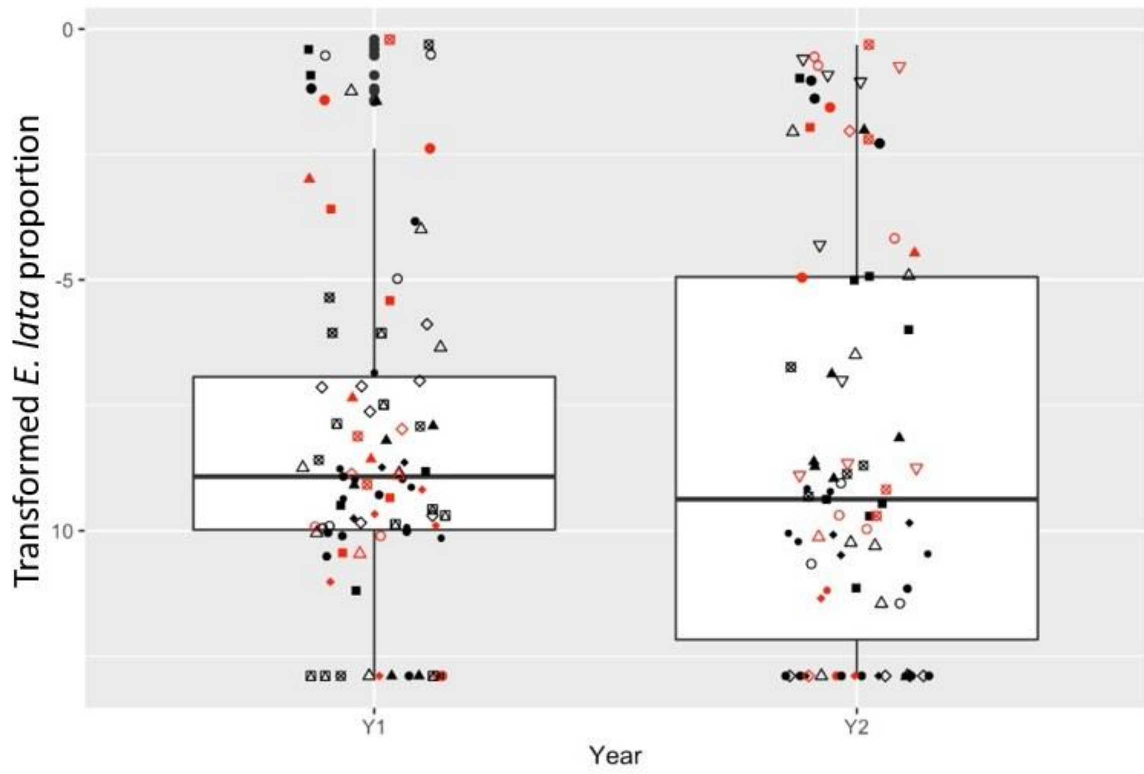
Question: Is the presence of symptoms influenced by Management, Variety, or Year, after controlling for Vineyard?

```
## Generalized linear mixed model fit by maximum likelihood (Laplace
##   Approximation) [glmerMod]
##  Family: binomial  ( logit )
## Formula: Symp ~ Management + (1 | Vineyard)
##    Data: loci3
##
##      AIC      BIC   logLik deviance df.resid
##    210.8    220.3   -102.4    204.8      175
##
## Scaled residuals:
##     Min      1Q  Median      3Q     Max
## -0.8859 -0.6290 -0.4812  1.1288  2.5031
##
## Random effects:
##  Groups   Name        Variance Std.Dev.
##  Vineyard (Intercept) 0.4008   0.633
## Number of obs: 178, groups:  Vineyard, 12
##
## Fixed effects:
##             Estimate Std. Error z value Pr(>|z|)
## (Intercept)  -1.2247     0.3701  -3.309 0.000937 ***
## ManagementF   0.3389     0.4939   0.686 0.492586
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Correlation of Fixed Effects:
##             (Intr)
## ManagementF -0.708
```

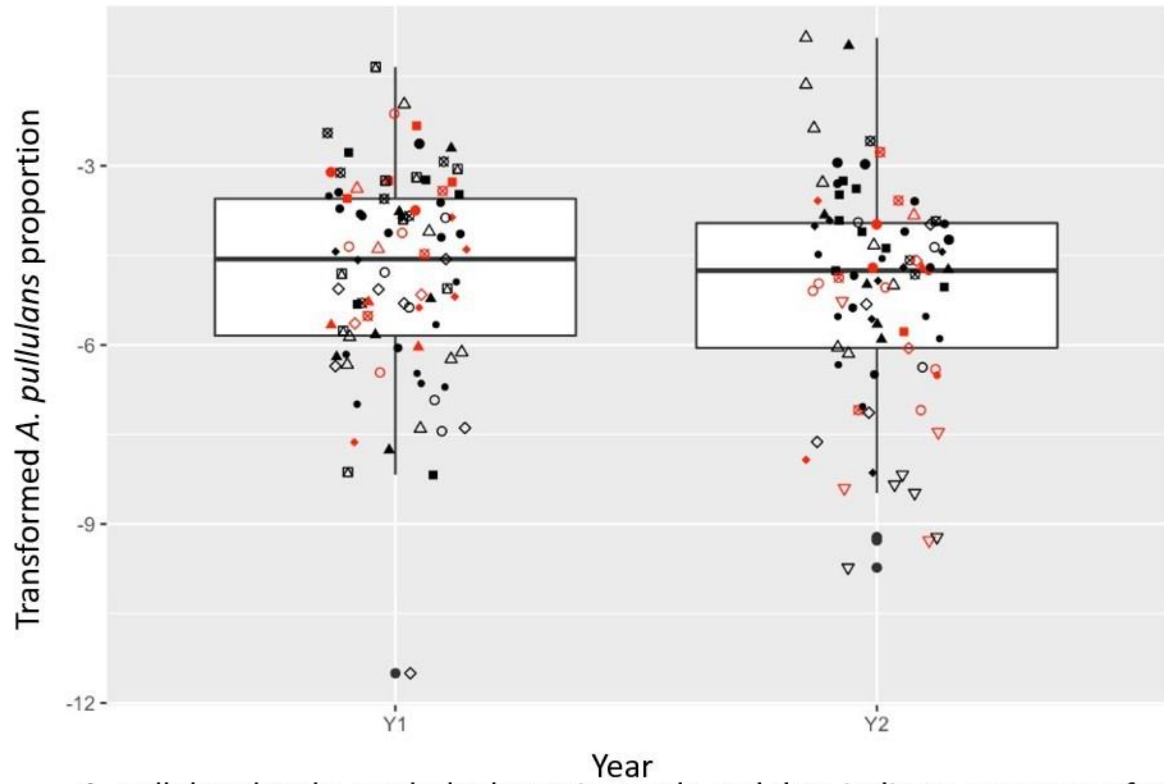
Conclusion: None of year, variety, or management appear to play a role in the presence of symptoms.

Question: Do the levels of *E. lata* (using the original transformation) and *A. pullulans*, and their interaction, predict the presence of symptoms, controlling for Vineyard?

Conclusion Neither *E. lata* or *A. pullulans* is associated with symptoms, either singly or in combination, when year 1 and 2 are looked at together. You will recall *E. lata* was associated with symptoms in year 2 (recreated below). This may be due to the increased variance of *E. lata* in this year (some vineyards low, some high). *A. pullulans* is not associated with symptoms in year 2; there is not sufficient sample size to fit them in combination.



E. lata levels: symbols show vineyard; red indicates presence of symptoms



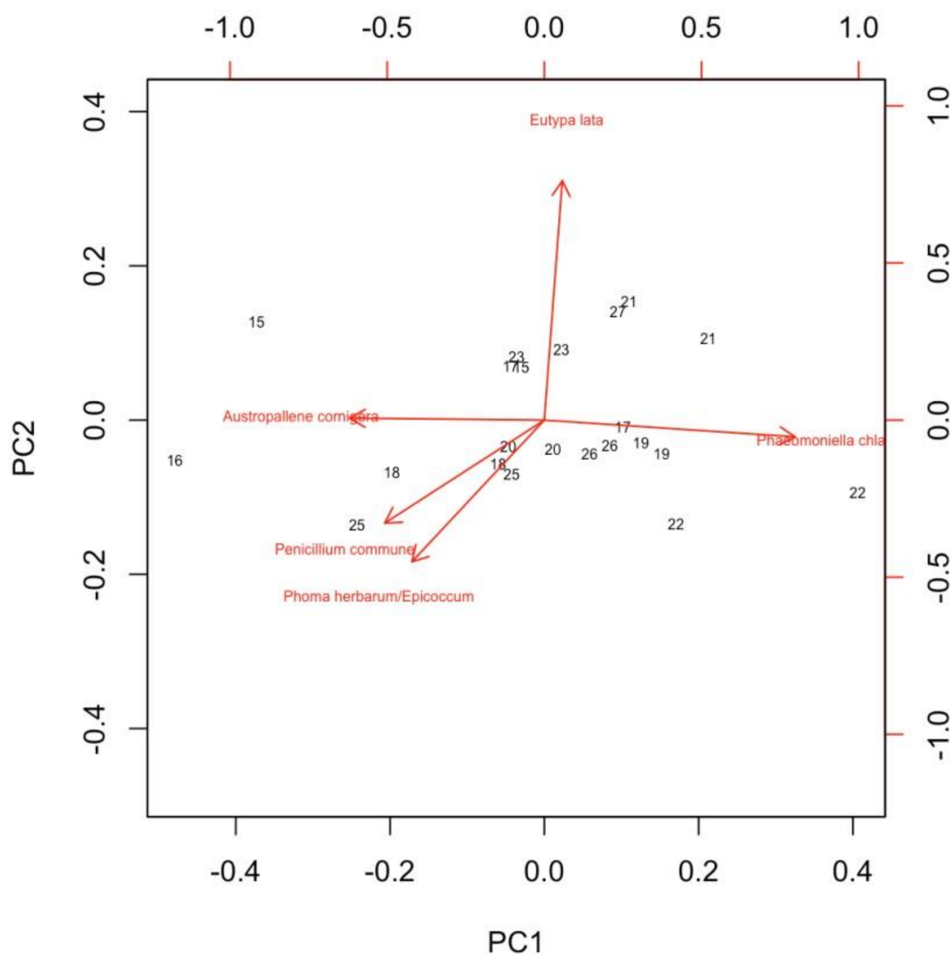
A. pullulans levels: symbols show vineyards, red dots indicate presence of at least one symptom.

3.3 Data analysis 2 from Dr Beatrix Jones (6 pages):

Changes in fungal profile due to year, management, and variety.

OTU counts have been aggregated to species level, then converted to proportions. A summary of the vineyard profile in each year is obtained by averaging over loci. Profiles are visualized using principal component analysis. A 79% of the variability in the data is accounted for by the first two principal components. The loadings indicate the first axis can be thought of as a contrast between *Phaemoniella chlamydospora* and *Astropallene cornisera*, with high *Phaemoniella* levels on the right and higher *Astropallene* levels on the left. The second axis reflects the *Eutypa lata* proportion. *Penicillium commune* and *Phoma herbarium* are also well represented in this space, with higher levels in the lower left quadrant.

Figure 1. Visualization of the top 5 principal component loadings. Numbers represent vineyard numbers.



We then test for differences in profile using the method of McArdle and Anderson (2001) as implemented in the R package "vegan" (2019). We have based our analysis on the euclidian distance between vineyard profiles to correspond to visualizations with principal In each case 999 permutations are used to assess significance.

We first test for differences over year, using vineyard as a strata. This essentially produces a paired comparison. There are significant differences between year (p=0.002). These are visualized in figure 2. For most vineyards, year 2 is to the right of year 1, indicating more *Phaeomoniella chlamydospora*. Year 2 is also frequently above year 1, indicating more *Eutypa lata* and less *Phoma herbarum*.

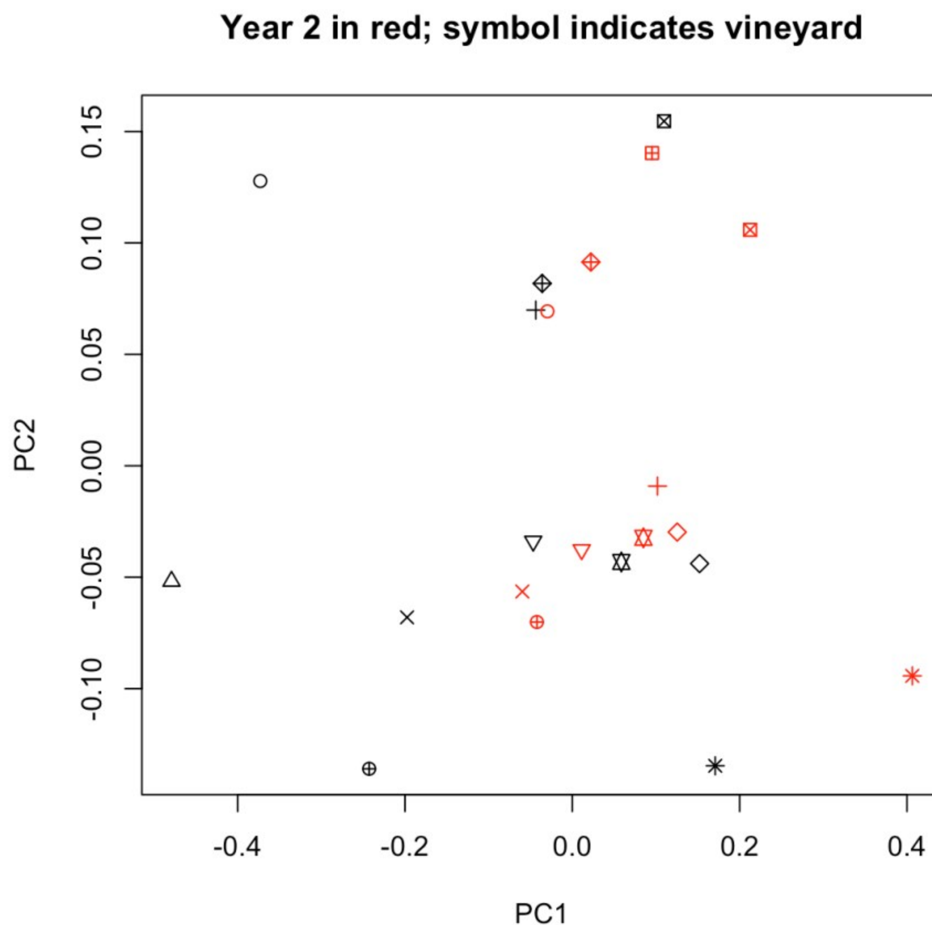


Figure 2. Visualization of inter-year differences.

We then test for the effects of variety, management, and their interaction, (sequentially, type I sum of squares) controlling for year (year used as a strata variable). Variety and management are both significant (p=0.011, p=0.006), but their interaction is not (p=0.167). The nature of the differences is not clear from visualizations. So individual linear models were fit to the dominant taxa *Phaemoniella* and *Eutypa*. These indicate *Phaemoniella* differs across variety (p=0.012) and management (p=0.018) after controlling for year, with Sauvignon Blanc and Future management having more *Phaemoniella*. *Eutypa lata* did not show differences by management or variety in these vineyard level aggregates; because *Eutypa* levels are lower, with many loci not showing any *Eutypa*, we considered a locus level model with a transformation of *Eutypa* abundance proportions.

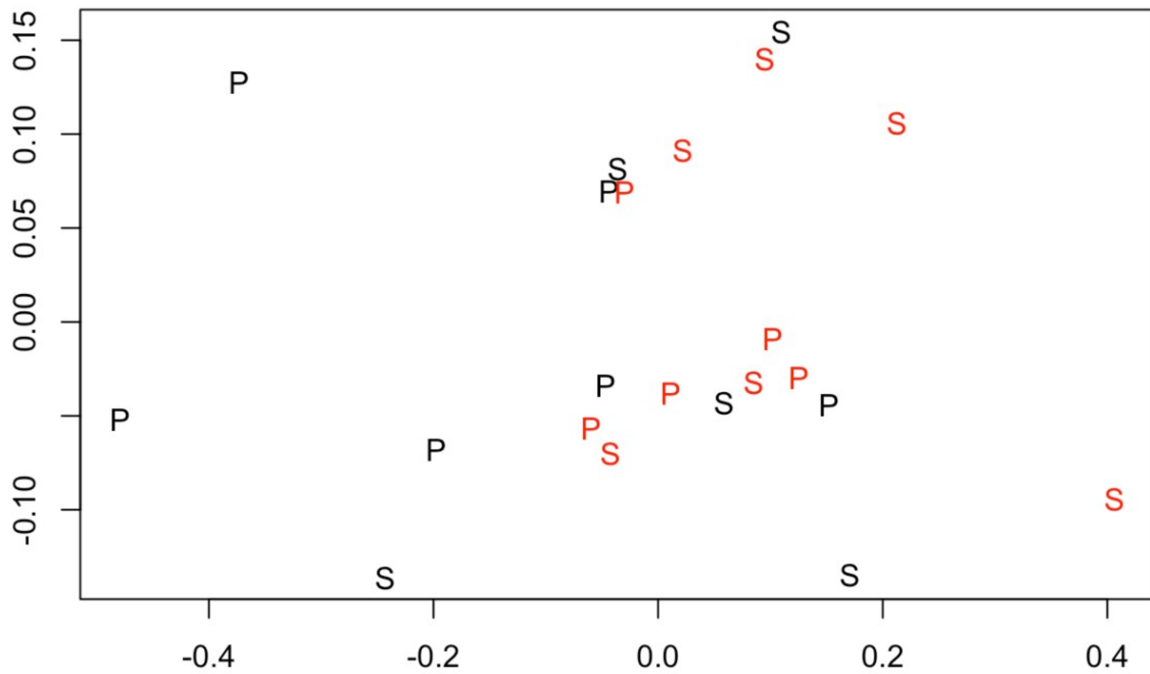


Figure 3. Principal component plot showing variety.

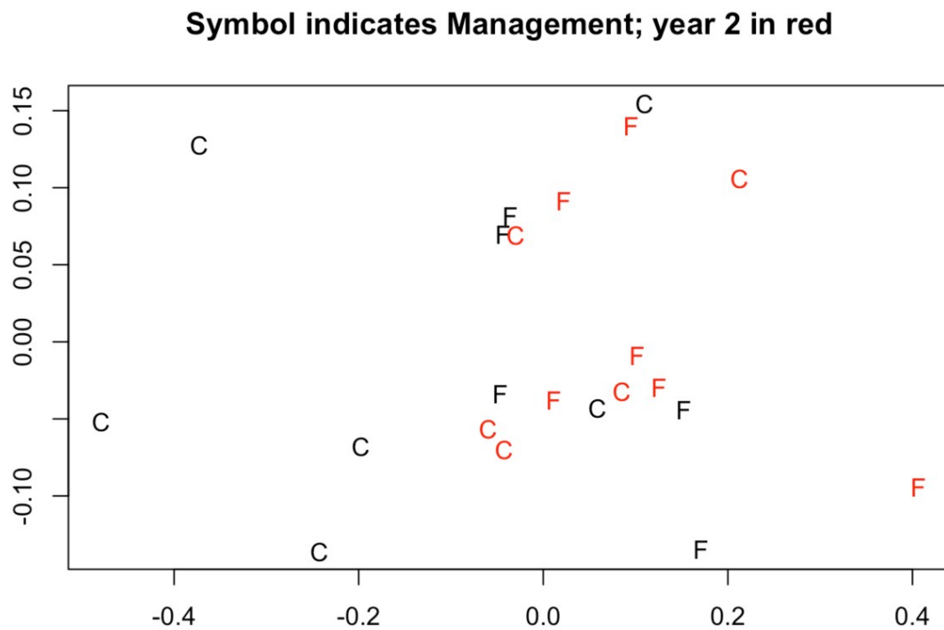


Figure 4. Principal component plot showing management.

Eutypa measurements at the locus level were transformed as log (abundance proportion+ $2.5*10^{-6}$). The constant was chosen as half the lowest observed species proportion; sensitivity analysis appears in the appendix. A linear mixed model was fit using the lme4 package (2015). Vineyard was treated as a random effect, and year, management, and variety as a fixed effects. Backward selection was performed based on likelihood ratio tests, with the criteria p=0.1 for retention of a predictor. Management was removed (p=0.51); Year and Variety were retained (p=0.07, p=0.02 respectively).

Associations between *Eutypa lata* and *Aureobasidium pullulans*.

There is particular interest in the interaction of these two fungi. *Aureobasidium pullulans* is ubiquitous across loci, though at varying levels. We log transform its abundance proportion and consider how it is affected by year, variety and management. We follow the same procedure of fitting a model with all these factors and performing backward selection. Year is eliminated (p=0.77) but Variety and Management are retained (p=0.01 and p< 0.001 respectively), with future management and Savignon Blanc vineyards having in less *A. pullulans*.

A model comparison assessing the association between the transformed abundance proportions of *Eutypa lata* and *Areobasidium pullulans* is then performed using the likelihood ratio test, and found to be significant (p=0.002). The baseline model includes vineyard as a random effect and all of Year, Variety and Management to ensure these potential confounders are controlled for. This is compared to a model that uses these factors and the transformed *Areobasidum* proportion to predict *Eutypa lata*.

Associations between Symptoms and Fungal profile, Variety, Year, and Management.

Initially, symptoms for a locus were considered to be the presence of canker, half head, stunted vines, or spur dieback; or the presence of young vines, retrunked vines, gaps, or dead vines. There are 48 loci with symptoms under this definition a generalized linear mixed model was fit for the presence of symptoms, using vineyard as a random effect, and doing backward selection on the predictors Year, Variety, and Management. None of these were significant (p=0.72, p=0.84, and p=0.49 respectively). Associations between symptoms and the fungal profile were considered. The effects on the fungal profile of year, management and variety were controlled for by stratifying using vineyard and year. The fungal profile did not differ between symptomatic and asymptomatic vines (permanova p=0.53). Individual assocations with *Eutypa lata* or *Aureobasidium pullulans* were also insignificant (p=0.29 and p=0.85 respectively, using a generalized linear model with the presence of symptoms as the response, and vineyard as a random effect).

Following analysis that showed that the half head and stunted symptoms were most predictive of vine mortality, these analysis were repeated considering only these two phenomena as symptoms. Only 16 loci show symptoms under this definition. None of Year (p=0.94), Management (p=0.37), Variety (p=0.28) , or *Aureobasidium pullulans* (p=0.84) are significant predictors. Symptoms are significantly associated with the transformed abundance proportion of *Eutypa lata* (p=0.033), although this conclusion is sensitive to the choice of transformation. The permanova test of the entire fungal profile shows no significant difference between symptomatic and asymptomatic loci (p=0.40).

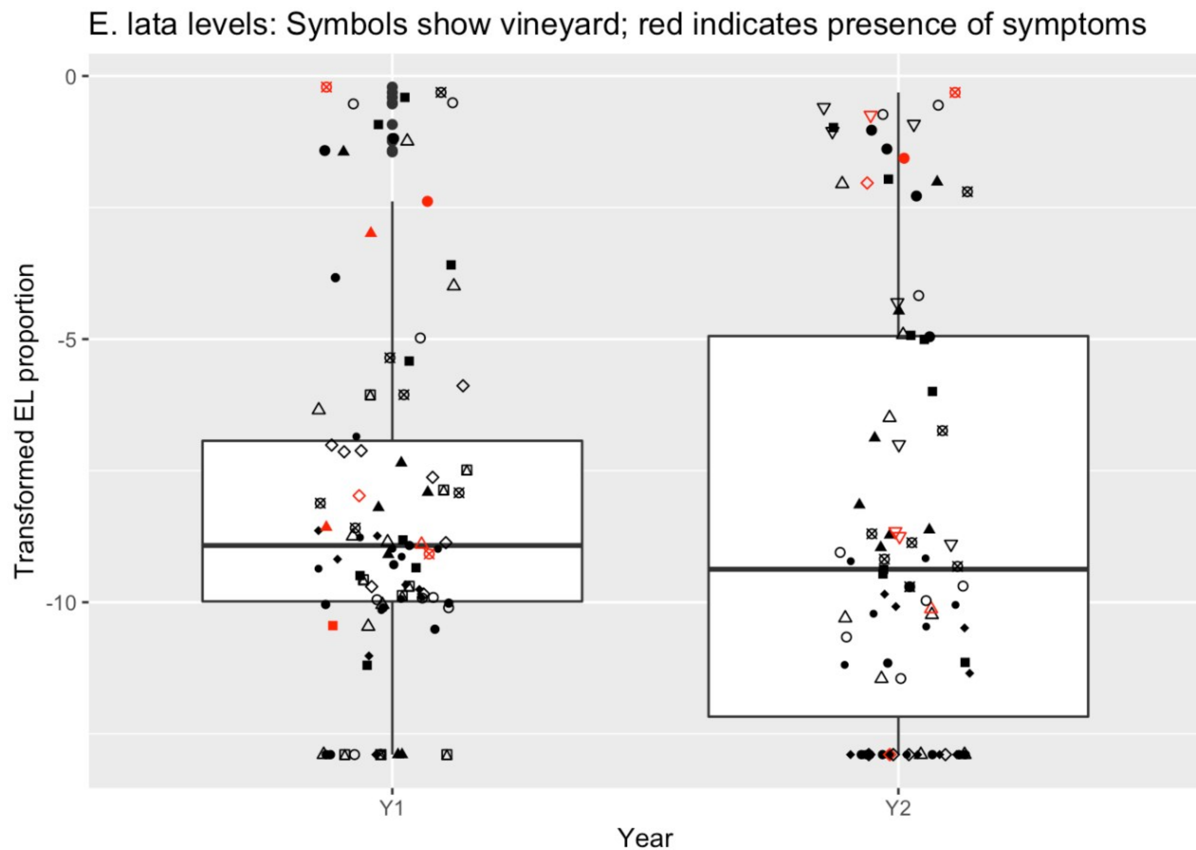


Figure 5: *Eutypa* levels and refined symptoms across years.

Appendix: Sensitivity analysis for *E. lata* transformation

We try two alternatives for the constant added before log transformation: 1.26×10^{-6} (half the current value), and 4.1×10^{-6}, half the minimum *E. lata* measurement (rather than half the measurement across the entire dataset).

The negative association with *A. pullulans* is robust to the choice of this constant (p=0.003 and p=0.001 under the alternate transformations.) The association of *E. latalata* with symptoms (under the refined definition) depends on the transformation chosen (p=0.11 and p=0.10 under the two alternatives considered.)

References:

McArdle, B.H. and M.J. Anderson. 2001. Fitting multivariate models to community data: A comment on distance-based redundancy analysis. *Ecology*, **82**: 290–297.

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Jari Oksanen, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs and Helene Wagner (2019). vegan: Community Ecology Package. R package version 2.5-4. https://CRAN.R-project.org/package=vegan

Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067.i01.

Table 3.3 *In vitro* Growth data and inhibitory rates of *A. pullulans* strains and *E. lata* strains (n=6). Either *A. pullulans* (one of four strains) and *E. lata* 20026, or only one were cultured on potato dextrose agar (PDA). Radius of colonies were measured when surface contact was first observed (10 dpi, or 10 dpi and 12 dpi for *A. pullulans* 20345).

Test group	strain	minimum radius (cm)	maximum radius (cm)	average radius (cm)	Standard deviation(σ)	Inhibitory rate (%)
Control groups	*E. lata* 20026	4.4	4.5	4.45	0.0548	
	A. pullulans 3057	2.1	2.4	2.30	0.1265	
	A. pullulans 21143	2.4	2.8	2.55	0.1643	
	A. pullulans 19713	2.3	2.6	2.53	0.1211	
	A. pullulans 20345	1.6[*1]/2.0[*2]	2.0[*1]/2.6[*2]	1.85[*1]/2.37[*2]	0.1378[*1]/0.1643[*2]	
Antagonistic groups						
E. lata 20026 and *A. pullulans* 3057	*E. lata* 20026	3.9	4.3	4.13	0.1506	7.2
	A. pullulans 3057	2.1	2.2	2.13	0.0516	7.4
E. lata 20026 and	*E. lata* 20026	3.9	4.5	4.10	0.2449	7.2

A. pullulans 21143	*A. pullulans* 21143	1.7	2.4	2.05	0.3209	10.9
E. lata 20026 and	*E. lata* 20026	3.8	4.1	3.93	0.1033	11.7
A. pullulans 19713	*A. pullulans* 19713	2.4	2.6	2.52	0.0753	0.4
E. lata 20026 and	*E. lata* 20026	3.3	3.8	3.48	0.1835	21.8
A. pullulans 20345*1	*A. pullulans* 20345	1.8	2.0	1.85	0.1517	0
E. lata 20026 and	*E. lata* 20026	4.0	4.4	4.25	0.1379	4.5
A. pullulans 20345*2	*A. pullulans* 20345	1.9	2.3	2.13	0.1506	10.1

4. Data of results from antagonistic tests

4.1 *In vitro* results of average colony radii and standard deviation

Table A4.1. Average colony sizes of *Aureobasidium pullulans* and *Eutypa lata in vitro* (n=6). Either *A. pullulans* (one of four strains) and *E. lata* 20026, or only one were cultured on PDA medium. Radii of colonies were measured when surface contact was observed at 10 dpi or both 10 dpi and 12 dpi for *A. pullulans* 20345. The *1 represents colony status at 10 dpi and the *2 represents colony status at 12 dpi.

Fungal treatment	Colony radius (standard deviation)

A. pullulans	*E. lata*	*A. pullulans* (cm)	*E. lata* (cm)
3057	20026	2.13 (0.05)	4.13 (0.15)
3057	-	2.3 (0.12)	-
19713	20026	2.51 (0.07)	3.93 (0.10)
19713	-	2.53 (0.12)	-
20345[*1]	20026	1.85 (0.15)	3.48 (0.18)
20345[*1]	-	1.85 (0.13)	-
20345[*2]	20026	2.13 (0.15)	4.25 (0.14)
20345[*2]	-	2.36 (0.20)	-
21143	20026	2.05 (0.32)	4.10 (0.32)
21143	-	2.55 (0.16)	-
-	20026	-	4.45 (0.05)

Table A4.2. Growth data and inhibitory rates of *Eutypa lata* and *Botrytis cinerea* on plates (n=6). Data was recorded at day 5 when the colony contact between *E. lata* and *B. cinerea* on plates was identified.

Test group	strain	minimum radius (cm)	maximum radius (cm)	average radius (cm)	Standard deviation (σ)	Inhibitory rate (%)
Control groups						
	E. lata 20026	1.5	2.0	1.82	0.2317	0
	B. cinerea	2.1	4.6	3.5	0.9920	0
Antagonistic groups						
E. lata 20026 and *B. cinerea*	*E. lata* 20026	1.5	2.3	1.83	0.3077	-0.55
	B. cinerea	3.6	4.5	4.2	0.3406	-20