STANDARDISED PRODUCTION OF AEROBIC COMPOST EXTRACT FOR DISEASE MANAGEMENT IN SUSTAINABLE VITICULTURE

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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July, 2009

DECLARATION

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ABSTRACT

Disease management in conventional viticulture involves regular applications of synthetic fungicides. There is, however, significant pressure from grape and wine markets to reduce inputs of synthetic fungicides because of concern about their safety to humans and the environment, and due to increasing evidence of pathogen populations developing fungicide resistance. Aerobic compost extract (ACE) is an oxygenated watery extract of compost that favours the persistence of aerobic microorganisms extracted from compost. Some horticultural practitioners claim that ACE is a sustainable alternative to synthetic fungicides when applied to the soil or to the fruit and foliage for crop protection. Scientific evidence supporting the effectiveness and safety of ACE is limited. Moreover, wide variation in production systems for ACE has made comparison of the few refereed reports available difficult. The primary aim of this research was to standardise production of ACE for safety to humans, and for consistent and high levels of suppression of two grapevine diseases caused by fungal pathogens of different biology; namely, powdery mildew, caused by *Erysiphe necator*, and botrytis bunch rot, caused by *Botrytis cinerea.*

Production variables for ACE from three composts with variable raw ingredients were evaluated systematically by quantifying the growth and reproduction of *B. cinerea* on detached bean leaflets treated with different ACEs. Bacterial-dominant ACEs produced with a compost weight to water volume ratio of 1:3 to 1:10, and from compost sampled in the very early secondary mesophilic stage of composting inhibited *B. cinerea* colonisation of bean leaflets to a greater extent than ACEs produced from compost sampled in the later mesophilic stages. There was evidence to suggest that the magnitude of pathogen suppression was associated positively to the number of bacterial and fungal Terminal Restriction Fragments (T-RFs) or

microbial taxon diversity in ACE. This association will need to be tested further by measuring T-RFs in ACEs prepared from a variety of compost windrows. ACE directly inhibited the germination of *B. cinerea* conidia *in vitro* and removal of microorganisms from ACE by filtration reduced but did not eliminate its capacity to inhibit conidial germination. Water-soluble antibiotics were not detected in filtered ACE following an *in vitro* assay for the inhibition of *B. cinerea* colony growth.

Under glasshouse conditions, the mean powdery mildew severity on Cabernet Sauvignon leaves was less than 0.1% when ACE was applied up to 4 days before or up to 7 days after inoculation with *E. necator* conidia; mean severity on non-treated, inoculated leaves was 22%. This result suggested that ACE had curative as well as protective properties. ACE or ACE amended with fish hydrolysate and/or liquid kelp was prepared using standardised methods and applied nine or 12 times at 10-14 day intervals to Chardonnay or Riesling vines grown commercially in different growing seasons in southern Tasmania. Powdery mildew was controlled by ACE or amended ACE to a commercially acceptable level on Chardonnay leaves and bunches under conditions of high disease severity. The incidence of latent *B. cinerea* in Chardonnay bunches at harvest, after moist incubation, was nearly half that observed in non-treated bunches. The incidence and severity of sporulation of *B. cinerea* on Riesling grape bunches was reduced significantly by ACE or amended ACE relative to a dechlorinated water control treatment; these control bunches (not leaves) escaped visible infection by *E. necator* but powdery mildew was controlled on leaves treated with ACE or amended ACE. Treatment of Riesling leaves with ACE increased the number of culturable microorganisms on leaves 100-fold, 1 h after application. By 13 days post-application the number of culturable microorganisms remained higher than pre-application counts.

The human pathogenic bacteria *Escherichia coli, Listeria monocytogenes* and *Bacillus cereus* were not detected in compost used to prepare standardised ACE. Production conditions for ACE did not favour re-growth of a non-pathogenic streptomycin resistant strain of *E. coli.* However, there was an increase in *E. coli* numbers when fish hydrolysate or molasses were introduced to ACE. Further experimental work is necessary to ensure negligible growth and persistence of human pathogens in standardised ACE amended with nutrients. In the interim, standardised ACE should be prepared without the addition of nutrients to prevent danger to human health.

The effectiveness of standardised ACE can now be evaluated across a range of viticultural conditions and for its impact on grape and wine quality. It is envisaged that ACE will be integrated with other measures to reduce the severity of diseases of grapevines and other horticultural crops.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my co-supervisor Kathy Evans for her constant support, encouragement and guidance throughout my Ph. D. Thank you also to Tom Ross for his extensive knowledge on microbiology and human pathogens and to my research supervisor Dean Metcalf for his advice on experimental design, *Botrytis cinerea*, bunch rot and sclerotia!!

I was extremely privileged during my Ph. D. to go to Europe and meet several of my idols including Jaak Ryckeboer, Heinrich Weltzien, Andreas Traakner and Nobel Ketterer. While writing my literature review I read many of their publications and to meet them and obtain guidance from them was such an honour.

I was also privileged to have several people at the University of Tasmania and New Town Research Laboratories help me with molecular biology and microbiology. In particular thank you to Janelle Brown for her assistance in experimental design for the human pathogen trials and to Fiona Poke, Allison Dann and Roger Latham for their continued support in the laboratories. Thank you also to Sharee McMannon, Shane Powell and Adam Smolenski for molecular assistance. Thank you very much to Steve Wilson and Ross Corkrey for statistical assistance. Thanks Justin Direen and Paul Schupp for your assistance in field work especially when the ATV kept breaking down and the smells, oh the smells!!! Also thank you to Bill Peterson for his handy work in setting up my spore settling tower and other equipment during my Ph. D., to Peter Crisp for advice on powdery mildew and organic viticulture and to the New Zealand crew (Joel Vanneste, Phil Elmer, Dion Mundy, Rob Beresford and Mike Spiers) for information on viticulture, bunch rot and organic fungicides.

I am very grateful to industry partner Soil First Pty Ltd, in particular David and Matthew Duggan. Thankyou for always having compost available when required. Also thankyou to Tony Scherer, Matthew Pooley and Margaret Pooley for allowing me to use their vineyards for field trials.

I would also like to express gratitude to the University of Tasmania, Tasmanian Institute of Agricultural Research and in particular the Australian Research Council for their important financial support.

Last and especially not least, thank you to all my friends and extended family who had to put up with my regular complaints and nagging over the last few years! In particular, thank you to Ange Smith for sharing a room with me, always there for a chat and taking me for sushi!!! Thanks to our little viticulture gang: Robbo, Fi, Reuban, Ange and Sam. Thank you Mum, Dad, G, D, Alex, Nan and Grandma for their relentless love, support and encouragement. Finally thankyou my gorgeous Andrew and my little Astro P for always keeping me positive and a smile on my face.

CONTENTS

PUBLICATIONS AND CONFERENCE PROCEEDINGS

Non-refereed journal article

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GLOSSARY (Definitions relating to fungi based on those of Hawksworth *et al.* (1995)

Term	Definition
Aerobic compost extract (ACE)	Compost extract produced to promote survival of aerobic microorganisms
Apothecium	A cup or saucer-like ascus
Appressorium	A growth on a germ-tube or hypha, for attachment in the initial stages of infection
Antagonism	A general term for interactions of organisms damaging to one or more of the associates i.e. antibiosis, parasitism
Ascomycete fungi	Division/Phylum of fungi, when reproducing sexually, produce non-motile spores in a cell called an ascus
Ascospore	A spore produced in an ascus by free cell formation
Ascus	A sexual spore bearing cell
Cleistothecium	A closed fruiting body of an ascomycete fungus in which ascospores are produced
Conidium	An asexual non-motile spore borne on a conidiophore
Conidiophore	Specialised hyphae bearing conidiogenous cells which produce conidia
Electrical conductivity	Soluble salt content
Flag shoots	Stunted shoots of grapevine that are covered partly or wholly by powdery mildew from buds infected by *Erysiphe necator* in the previous season before flowering. Most easily detected 2-6 weeks after budburst.
Hypha	One of the filaments of a mycelium
Mycelium	The vegetative body of a fungus; a mass of hyphae
Non-aerobic compost extract (N-ACE)	Compost extract produced without aeration
Sustainability	Using, conserving and enhancing the community's resources so that ecological processes, on which life depends, are maintained, and the total quality of all species life, now and in the future, can be maintained or increased
Sclerotia	A mass of vegetative hyphae, in or on which asexual spores or sexual fruiting bodies (eg. apothecia) are produced
Withholding period	Period that must elapse from last day of application of a pesticide until the first day of crop harvest

INTRODUCTION

Powdery mildew and botrytis bunch rot are two economically important diseases in grapevines worldwide (Pearson and Goheen 1988, La Guerche *et al.* 2006). Conventional viticulture aims to prevent these two diseases by regular applications of synthetic fungicides (Wicks *et al.* 2002). Aerobic compost extract (ACE) is a watery extract of compost, otherwise known as compost tea, which some practitioners claim is a sustainable alternative to synthetic fungicides. However, there has been little scientific research investigating the effectiveness and mechanism of ACE in suppressing foliar and fruit diseases. The primary aim of this research was to standardise production of ACE for maximum suppression of grapevine powdery mildew, caused by *Erysiphe necator*, and bunch rot, caused by *Botrytis cinerea.* Evidence is presented to demonstrate the potential for ACE to be integrated with other measures to reduce diseases of grapevines and other horticultural crops to a commercially acceptable level.

A diagrammatic representation of the thesis structure is presented in Fig. 1 to show how this thesis is divided into chapters that relate to specific hypotheses. The next section of this thesis reviews the theory and practice of aerobic compost extract (ACE) for disease management, and fruit and foliar disease control by application of ACE, with particular reference to powdery mildew and botrytis bunch rot of grapevine. The project aims are listed at the end of the review. In Chapter 1, a rapid bean leaflet bioassay was developed to test the ability of different ACEs to suppress colonisation and sporulation of *B. cinerea.* This bioassay revealed the parameters of ACE production that were the most important in suppressing *B. cinerea.* The diversity of bacterial and fungal taxa, determined by terminal restriction fragment length polymorphism (T-RFLP) was correlated with the suppressive ability of ACE.

Subsequently, a standardised, pathogen-suppressive ACE was used to determine the mechanism of pathogen suppression. In Chapter 2, having developed some understanding of the processes that regulate efficacy of ACE *in vitro,* the system was applied *in vivo* and in the field. Grapevines grown in the glasshouse were inoculated with *E. necator* conidia to assess the effectiveness of a single application of ACE in relation to the timing of pathogen infection and to explore whether ACE was acting to prevent infection or to limit pathogen colonisation. The field experiments illustrated the effectiveness of standardised ACE, and ACE amended with nutrients, for limiting natural epidemics of powdery mildew and botrytis bunch rot on grapevines in commercial production. The possibility of human pathogen presence and re-establishment in ACE was investigated in Chapter 3. Finally, Chapter 4 assembles the thesis results in a general discussion and presents the main conclusions. Chapter 1, 2 and 3 have been written in manuscript format.

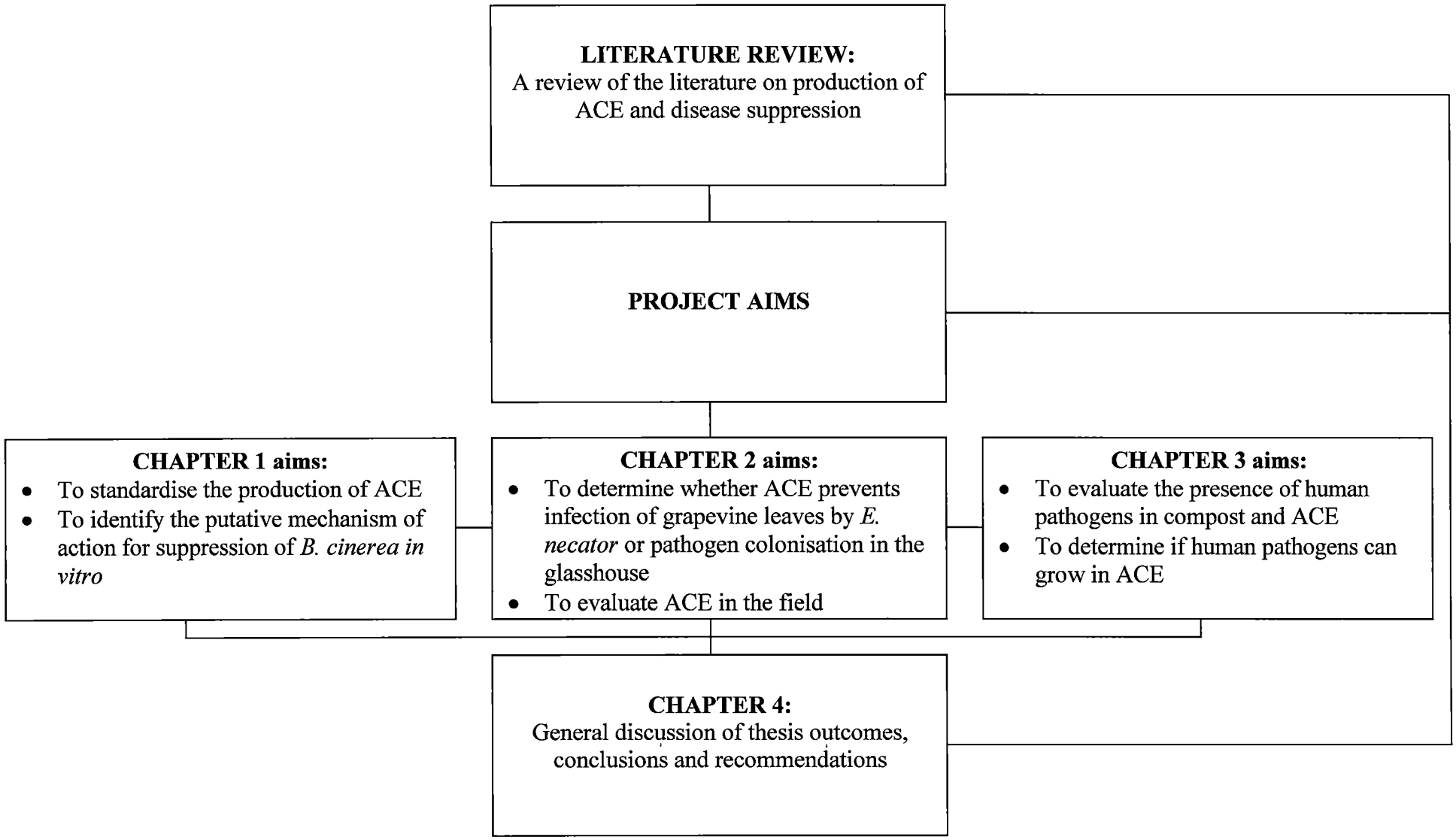


Figure 1 A diagrammatic representation of the structure and objectives of the thesis.

LITERATURE REVIEW

Introduction

Synthetic fungicides are a major component of conventional management of fungal diseases in viticulture. However, development of resistance in pathogen populations to certain synthetic fungicides (Steden *et al.* 1994, Erickson and Wilcox 1997, Savocchia *et al.* 2004) and public demand for residue-free wines (Kerr 1992) has led to a greater need to produce a sustainable solution to crop disease management.

A promising disease control practice that could form part of a sustainable, integrated management strategy is the application of aerobic compost extract (ACE). ACE is produced by leaching microorganisms and nutrients from aerobic compost into oxygen-rich water (Scheuerell and Mahaffee 2002). Practioners have made claims that ACE, when applied to the crop canopy, suppresses a broad range of diseases while maintaining biodiversity in an agricultural ecosystem (Riggil 1996, Touart 2000). There are very few scientific research papers illustrating disease control with ACE (Cronin *et al.* 1996, Welke 2004, Scheuerell and Mahaffee 2006), yet ACE is the most frequently recognised and employed method of compost extract production among practitioners today (Scheuerell and Mahaffee 2002, Litterick et al 2004). ACE has successfully replaced the production of non-aerobic compost extract (N-ACE) due to faster and more reliable extraction (Ingham 2003), a perceived absence of human pathogens and a lack of toxic metabolites from non-aerobic microorganisms (Ingham 2000).

There are a substantial number of variables involved in the production and application of ACE and several topics that require further research. Like any new crop protectant that is developed, a consistent product must be produced in order to

control disease effectively and reproducibly. Standardisation of ACE is likely to involve analyses of the compost source, extraction process and an understanding of mechanism of action and maintenance of microorganisms applied in the field. Previous research investigated some aspects of ACE standardisation (Welke 2004, Scheuerell and Mahaffee 2006) but progressive analyses of greater depth will be essential for commercialisation and development of a simple and reliable method of ACE production, while ensuring high efficacy as well as human safety.

The aim of this review is to identify research required to produce an ACE that is safe to humans and consistently suppresses two economically important diseases of grapevine, powdery mildew and botrytis bunch rot.

History and impact of powdery mildew and botrytis bunch rot in viticulture

Common grape, *Vitis vinifera,* is cultivated globally and is the most widespread grapevine belonging to the Vitaceae family. *V. vinifera* is cultured for table grapes or processed into wine, sultanas or juice (Pearson and Goheen 1988). As it is a perennial crop, research must be conducted over several seasons in order to obtain robust conclusions.

V. vinifera is susceptible to an extensive array of pests and diseases, particularly phylloxera, powdery and downy mildews, and bunch rots caused by various pathogens. Powdery mildew and botrytis bunch rot, caused by the fungal pathogens *Erysiphe necator* Schw. (Braun and Takamatsu 2000; synonym *Uncinula necator*) and *Botrytis cinerea,* are two important foliar and fruit diseases of grapevine that affect vineyards worldwide (Pearson and Goheen 1988, Gadoury *et al.* 2001). In the temperate, maritime climate of Tasmania, where relative humidity during the

growing season is often above 60%, powdery mildew develops in non-treated vines in most growing seasons (Scott *et al.* 2007). Botrytis bunch rot develops to varying degrees each season in susceptible grapevine varieties in Tasmania (K. J. Evans, personal communication). Delays in harvesting varieties with compact bunches, especially when crop loads are high, may increase the risk of a severe epidemic of botrytis bunch rot when there is sufficient moisture during the ripening period. Both diseases are managed in most Tasmanian vineyards with protective fungicides every growing season, which represents a significant cost to the grower.

Powdery mildew is one of the most damaging fungal diseases of grapevines globally (Gadoury *et al.* 2001) and can lead to chlorosis, necrosis and reduced vigour and productivity of grapevine. Infections on the young green leaves can lead to a reduction in photosynthesis (Nail and Howell 2005), and thereafter a decline in total soluble solids of berries (^{o}Brix) which is important in wine production (Stummer *et al.* 2005). Grapes with powdery mildew infection used for wine making have been shown to possess 'dusty' or 'mouldy' aromas, an 'oily' and 'viscous' component, and green, bitter and herbaceous tannins (Stummer *et al.* 2002, Emmett *et al.* 2004). Powdery mildew can make wine and table grapes unmarketable.

B. cinerea Pers. Fr. (telemorph *Botryotinia fuckeliana*) attacks many different plant species (Coley-Smith *et al.* 1980). Severe epidemics of botrytis bunch rot in grapevines cause significant crop and economic loss (Elmer and Michailides 2004). Infection of berries by *B. cinerea* can lead to an increase in laccase production, which reduces wine quality because phenolics such as the red colour compounds in red wine are oxidised to brown coloured compounds (Iland *et al.* 2000).

E. necator and *B. cinerea* have very dissimilar life cycles and ecological niches. *E. necator* is an obligate parasite that infects green tissue and young growth in grapevines and as a consequence must be cultured with its host plant (Evans *et al.* 1996), whereas *B. cinerea* is a cosmopolitan, necrotrophic pathogen that obtains nutrients from dead tissue of many plant species, including grapevines (Pearson and Goheen 1988). Both fungi have the ability to infect leaves, flowers and berries on *V. vinifera. E. necator* infection of grapevine berries is greatest at fruit set and resistance to *E. necator* increases with berry age (Ficke *et al.* 2002). In contrast, berry and flower resistance to *B. cinerea* decreases with tissue age (Jarvis 1977). Furthermore, berries can become highly susceptible to infection by *B cinerea* condia if wounded (Coertze and Holz 2002). The significant differences between these pathogens in life history and biology mean that the basis for their control is likely to be different.

Biology of *E. necator*

E. necator belongs to the Erysiphales and has a narrow host range (Agrios 1997). The pathogen is spread by windborne ascospores and/or conidia. *E. necator* can overwinter in dormant buds as mycelia, which generate conidia upon recolonisation of grape shoots in spring (Rumbolz and Gubler 2005) or may overwinter as cleistothecia that release ascospores following maturation (Gadoury and Pearson 1991). Conidia contribute to multiple infection cycles during the growing season. Environmental conditions favouring various stages in the life cycle of *E. necator* are described in Table 1. The key factors determining the onset and rate of development of epidemics include surface moisture for the release of ascospores, relative humidity, which influences the germination of conidia, and temperature, which determines the time from infection to sporulation. Nutrient supply and UV intensity

also affects powdery mildew development. Willocquet *et al.* (1996) showed that spore germination and mycelial growth decreased on grapevine leaf disks when exposed to UVB and that shaded or cloudy conditions were more favourable to spore germination and mycelial growth than sunny conditions. In potted grapevines, Keller *et al.* (2003a) illustrated that high nitrogen supply and low UV increased *E. necator* incidence and severity.

A diagram of the life cycle can be found in Pearson and Goheen (1988).

Table 1 Environmental conditions favouring various stages in the lifecycle of *Erysiphe necator* on a susceptible variety of *Vitis vinifera* (RH = relative humidity).

Stage of lifecycle	Experimental conditions	Optimum environmental conditions	References
Ascospore discharge and infection	Field: berries and leaves	> 2.5 mm rain	Gadoury and Pearson (1990)
		> 2.5 h wetness duration > 11°C	Jailloux *et al.* (1999)
	Field: leaves	12-15 h leaf wetness when average temperature 10-15°C	Gubler *et al.* (1999)
Ascospore germination	*In vitro*	Maximum germination at 25°C	Hajian-Shahri *et al.* 2006
Regrowth of dormant mycelium	Field: leaves	18-30°C	Gubler *et al.* (1999)
Germination of conidia	*In vitro*	85% RH	Carroll and Wilcox (2003)
		24-25°C	Delp (1954) Bendeck *et al.* (2007)
	Field: leaves	25°C	Gubler *et al.* (1999)
Conidial production	Field: leaves	7-10 days after primary infection	Gubler *et al.* (1999)
Rate of sporulation	Field: leaves	Three consecutive days with at least 6 h at 21-30°C	Gubler *et al.* (1999)

Biology of *B. cinerea*

B. cinerea can overwinter as sclerotia within bark or on decaying infected plant material, or as vegetative hyphae in dormant buds, leaves and canes on the vineyard floor or as remnants in the vine canopy (Elmer and Michailides 2004). In spring, sclerotia produce mycelia that bear conidiophores containing conidia or, when compatible sexual mating types unite, the sclerotium bears an apothecium containing asci (Elmer and Michailides 2004). *B. cinerea* conidia are the principal source of inoculum for infection of grape bunches (Nair & Nadtotchei 1987). Conidia are primarily dispersed by wind and water droplets (Holz *et al.* 2000) and occasionally by insects (Fermaud and Le Menn 1989). Environmental conditions that facilitate development of botrytis bunch rot in grapevine are illustrated in Table 2.

Table 2 Environmental conditions favouring various stages in the lifecycle of *Botrytis cinerea* in relation to fruit infection and development of bunch rot of a susceptible variety of *Vitis vinifera.*

Stage of lifecycle	Experimental conditions	Optimum environmental conditions	References
Appressorium formation	*In vitro*	15–20°C	Shiraishi *et al.* (1970)
Elongation of germ tubes	*In vitro*	25–30°C	Shiraishi *et al.* (1970)
Mycelium development	*In planta:* berries	21°C 94% RH 0 m/s wind speed	Thomas *et al.* (1988)
Colony expansion	*In vitro*	20–30°C Dark	Shiraishi *et al.* (1970)
Conidial production	*In planta:* berries	21°C 94% RH 0.6 m/s wind speed	Thomas *et al.* 1988
Symptom expression	*In planta:* flowers	23.7°C 1.3 h wetness	Nair and Allen (1993)
	In planta: berries	20.8°C 13.9 h wetness	Nair and Allen (1993)

There are various infection pathways for *B. cinerea* in grapes (Elmer and Michailides 2004). *B. cinerea* conidia usually infect soft or moist senescent tissues, especially ripe or decayed fruit, wounded tissue and dehiscent flowers. The temperature, humidity and duration of wetness that prevail during flowering, veraison and bunch closure will have a significant effect on the timing of *B. cinerea* infection and hence the infection pathway and disease management.

Disease management

Approaches to disease management in horticulture can be divided into three broad categories: (1) conventional, (2) organic and biodynamic and (3) integrated disease management. In conventional horticulture, disease is managed primarily using synthetic fungicides, whereas in organic and biodynamic systems, synthetic fungicides are omitted. Integrated disease management combines both organic and conventional methods of disease control. Regardless of production system, the horticultural sector is searching for the most sustainable approach to disease management.

(1) Conventional viticulture

A wide array of synthetic chemicals are used for managing grapevine diseases in conventional viticulture (Wicks *et al.* 2002) and each chemical targets the metabolism of a plant pathogen at critical points in its life cycle, during the host-pathogen interaction. Synthetic chemicals are classed into groups of fungicides based on the specific mechanism of action or the metabolic reaction targeted in the fungus or plant.

Resistance of fungal populations to synthetic fungicides is a major concern in conventional viticulture. The dimethylation inhibiting fungicides (DMIs), for example, are losing effectiveness for controlling powdery mildew in the field in California (Ypema *et al.* 1997, Gubler *et al.* 1996), Portugal (Steva 1994) and Australia (Savocchia *et al.* 2004). In Australia, the Agricultural and Veterinary Chemical Control of Use Act regulate product labelling of synthetic fungicides. Label instructions help prevent fungicide resistance and relate to length of spray intervals, spray dose, maximum number of applications per growing season, the maximum residue limit (MRL) in wine and the withholding period. The extent of restriction, especially for wine destined for export to countries that have a very low or no MRL, can make it difficult to control several grapevine diseases. For example, there are limited synthetic botryticide options available to control botrytis bunch rot during the pre-harvest period (Bell and Essling 2007), and at this time, berries are most at risk of *B. cinerea* infection due to increasing berry age.

(2) Organic and biodynamic viticulture

In contrast to conventional viticulture, non-synthetic chemicals and labour-intensive monitoring for pests and diseases replace calendar-based application of synthetic chemicals. Essentially, organic viticulturalists need to understand the epidemiology and life cycle of pathogens for successful disease control. Disease can be limited if the crop is manipulated to alter host susceptibility or microclimate. Cultural control in this way can reduce reliance on synthetic fungicides. Organic growers must integrate knowledge of disease susceptibility of each grapevine variety (Boso *et al.* 2004) and the impact of irrigation practices (Keller *et al.* 2003b), nutrition (Cole 2002), canopy management (Cole 2002) and biological fungicides (Elmer and Reglinski 2006) when managing powdery mildew and botrytis bunch rot.

During the growing season, powdery mildew may be controlled effectively by regular applications of elemental sulfur. Even though sulfur sprays are "certified" as organic, there is anecdotal evidence to suggest that vineyard workers and neighbours can develop allergies to sulfur. As stated previously, sulfur can be toxic to beneficial insects (Calvert and Huffaker 1974) and phytotoxicity is considered a risk when temperatures exceed 40°C and relative humidity is above 70% (Magarey *et al.* 2002). Therefore, alternatives are suggested because this method of disease control may not be sustainable. Potassium bicarbonate, oils, milk, whey and biological control agents have been tested on grapevines as alternative methods for powdery mildew control (Azam *et al.* 1998, Yildirim *et al.* 2002, Crisp *et al.* 2003, Scott *et al.* 2007). Biological control agents and ACE have been evaluated as alternative methods for botrytis bunch rot control (Dubos 1992, Elad and Shtienberg 1994). Most grape growers need to be convinced about the effectiveness and reliability of alternative measures before adoption, which becomes a challenge when the test materials are not well understood and/or the experimental results are highly variable. Research on biological control and ACE is reviewed later in this section.

Biodynamic viticulture concerns disease management techniques, which are viewed by many as having a "spiritual" component (Moore 1997). The modern concept of biodynamics originated in the early 1920s when a number of lectures were given by Rudolf Steiner, a philosopher from Austria (Steiner 1974). The specific seasons, the moon and stars are all important in biodynamic agriculture. Compost is also regularly applied to biodynamic crops. Scientific verification of biodynamic viticulture and agriculture is lacking, for example, Carpenter-Boggs *et al.* (2000) compared soil microbial biomass and activity in a randomised complete block experiment on two

broad-acre farms and illustrated no significant difference between biodynamic and organic fertiliser regimes. In fact, there is a lack of research investigating the difference between conventional and organic/biodynamic practices and their effect on microbial ecology of the soil and/or aerial plant surface.

(3) Integrated disease management

Since the early 1990's, Australian viticulture has adopted, at least in theory if not practice, the principles of integrated disease management (IDM). IDM aims to prevent disease outbreaks by understanding the life cycles of arthropods and pathogens in relation to the host plant and the environment. Cultural controls, such as canopy or vineyard floor management, are integrated to reduce sources of inoculum and/or the infection efficiency of the pathogen. If disease cannot be prevented by cultural and/or biological controls then synthetic fungicides are utilised to prevent loss of crop yield or quality. Additionally, prediction or forecasting tools can aid IDM practitioners to time application of synthetic fungicides strategically, according to a susceptible crop stage and/or availability of pathogen inoculum.

Biological control agents

Various single microorganisms have been demonstrated to suppress the grapevine pathogens *B. cinerea* (Elad 1996, Reglinski *et al.* 2005) and *E. necator* (Falk and Gadoury 1994, Falk *et al.* 1995). Many biological control agents have been reported to be as effective as synthetic chemical control under glasshouse and controlled environmental systems (Elad *et al.* 1993, Dik and Elad 1999, Guetsky *et al.* 2001). Currently, the highest percentage of biocontrol agents used commercially in horticulture is from the two genera, *Trichoderma* and *Bacillus* (Stewart 2001). There are several mechanisms by which species of *Trichoderma* reduce pathogen infection

including parasitism of pathogenic fungal mycelia, antibiosis and/or production of cell wall degrading enzymes such as chitinases and β–1,3–glucanases (Elad 1994, Metcalf 2002). Research on the biological control potential of *Trichoderma* spp. against *B. cinerea* on grapevine began in the late 1970's (Dubos *et al.* 1978, 1982) and the greatest reduction in disease incidence and severity to date have been under low to moderate disease pressure (Bisiach *et al.* 1985, Gullino and Garibaldi 1988, Garibaldi *et al.* 1989).

Single biological control agents that perform well in controlled environments often produce inconsistent results in the field, which suggests that single species are constrained by mechanism of action, and/or abiotic conditions such as temperature, humidity and rainfall. Several authors discuss application of two or more biological agents to increase disease suppression (Guetsky *et al.* 2002, Stewart 2001). The mechanism of action of multiple biological control agents may be additive and disease control improved in a variable cropping environment. Another strategy is to combine biological control with other control methods. In glasshouse and field experiments with grapevines, Reglinski *et al.* (2005) improved the efficacy of a single biological control agent, *Ulocladium oedemansii,* by applying the fungal antagonist in combination with a chemical elicitor of induced plant resistance (5-chloro salicylic acid (5-CSA)). Treatment with both *U. oedemansii* and 5-CSA was as effective as treatment with 0.1% Shirlan® (fluaziman) for preventing *B. cinerea* sporulation on grapevine bunches. This approach to disease management shows great potential; however, the large number of applications would not be an economically viable option for grape growers when compared to synthetic fungicides. Furthermore a second year of field data by Reglinski and colleagues has demonstrated a reduction in berry weight, leaf chlorosis and residues in wine following regular application of

5-CSA. (T. Reglinski, HortResearch New Zealand, personal communication). These results illustrate that it is important to measure grape and wine quality when testing developmental materials and to conduct field experiments over several seasons.

Aerobic compost extract (ACE)

History and production techniques

Production systems for compost extract have predominantly been described in the literature as non-aerobic or aerobic (Litterick *et al.* 2004). In non-aerobic systems, compost is left soaking in water for several days before filtering to produce an extract (Fig. 2A). In contrast, compost in aerobic systems is generally held in a porous screen in dechlorinated water and a circulating water pump or stirring is applied to maintain aerobic conditions for 24 to 72 h (Fig. 2B).

Production of non-aerobic compost extract (N-ACE) has been performed for centuries. Ancient Egyptians, Greeks and Romans were the first to suspend bags of manure in drums of water to produce a leachate (Ingham and Alms 2003). These N-ACEs were presumably applied to crops to improve plant growth. It was not until the 1920s, however, that organic farmers recognised the disease suppressive qualities of N-ACE (Brinton 1995). Disease suppression was thought to be due to antagonistic effects of microorganisms present in N-ACE (Brinton 1995).

A	B
Mix 1 part compost to 5–10 parts tap water (w/v)	Place compost in a porous bag/container and suspend in dechlorinated water as follows: 1 part compost to 4 parts water (w/v)
↓	↓
Leave at ambient temperature for 5–10 days	Maintain aerobic conditions (> 6 mg / L dissolved oxygen) and leave for 24–72 h at 22°C ± 2°C
↓	↓
Filtration through cheesecloth or other filters	No filtration required
↓	↓
Apply with standard equipment as a fungicide or for fertigation	Apply with standard equipment as a fungicide or for fertigation

Figure 2 Flow diagrams of compost extract production systems. A. A non-aerobic system for extract production (Weltzien 1992). B. A generalised production system for aerobic compost extract.

Scientific studies on N-ACE began in laboratory and greenhouse settings, with the first experimental results reported by Weltzien and Ketterer (1986). Preliminary bio-assays demonstrated suppression of downy mildew on glasshouse grown grapevine leaves when N-ACE was applied as a foliar spray to the abaxial surface of detached leaves prior to inoculation with *Plasmopara viticola* (Weltzien and Ketterer 1986). Weltzien (1989) was the first to suggest N-ACE as an economically viable alternative to synthetic fungicides but recommended further research.

Researchers have studied production techniques for N-ACE more extensively than ACE production techniques for variables such as ingredients used to initiate composting, duration of compost extraction and the influence of adding nutrients to the extracts (Weltzien 1990, 1992, Ketterer *et al.* 1992, Scheuerell 2003). These studies have been conducted *in vitro,* on detached leaves, in glasshouse and field environments, and the majority of experiments have illustrated statistically less disease in the N-ACE treatments than the water or non-treated control treatments. In

controlled environments, N-ACE was just as effective as the synthetic fungicide treatments (Weltzien 1989). Unfortunately, very few of these published reports stated the disease severity in field experiments, which is essential in viticultural studies because as little as 4% severity of powdery mildew or botrytis bunch rot can cause winemakers to apply a price penalty to harvested grapes (Cooperative Research Centre for Viticulture 2005). The benefit of the early research on N-ACE was that it highlighted the variables associated with extract production, such as compost source, extraction times and method of application. The detached leaf bio-assay of Weltzien and Ketterer (1986) illustrated the type of rapid technique needed to reliably evaluate different ACEs prior to testing in the glasshouse or field.

Only since the mid-1990s have researchers and practitioners been considering aerobic systems for extract production. ACE has replaced N-ACE due to frequent grower reports of fungal disease control, considerably faster production times and because there are now many companies promoting and selling ACE. Most studies investigating ACE have been performed in the field. The focus of research has been to compare (1) ACE to N-ACE (Welke 2004, Scheuerell and Mahaffee 2006), (2) unamended ACE to ACE with nutrients (Cronin *et al.* 1996, Al-Dahmani *et al.* 2003, Scheuerell and Mahaffee 2004), (3) various dilutions of ACE (Welke 2004, Scheuerell and Mahaffee 2004) and (4) unamended ACE to ACE with additional spreaders/stickers (Scheuerell and Mahaffee 2006). The percentage of disease control within each study has been conflicting and the majority of researchers have concluded that the ACE is an ineffective means of disease control. It is clear that there has been a lack of standardisation of ACE production among studies.

Considering the increase in ACE production and application globally, plus the shortage of detailed research papers illustrating disease control by ACE; the next section of this review focuses on the variables associated with ACE production and which conditions might be standardised before testing ACE in the field.

Factors affecting ACE quality

Source of compost

Many filamentous fungi, bacteria, nematodes and yeasts are present in composted material (Weltzien 1992) and the diversity, abundance and activity of microorganisms varies according to compost source and maturity (Hoitink and Boehm 1999, Noble and Roberts 2003). Compost of high quality, as defined in Table 3, might be necessary for maximum disease control by its extract, although this hypothesis would need to be tested empirically. The quality of disease control by ACE may depend on compost age, type (animal, plant material), carbon to nitrogen ratio (C:N), pH, temperature, moisture content and nutrient levels.

Table 3 Typical ranges of test parameters for compost prepared according to the Australian Standard for compost (AS 4454 2003).

Parameter	Measure	Range
pH		5–7.5
Electrical conductivity		No limit, although high EC can be detrimental to crop production
Soluble phosphorus	mg/L	≤ 5 for phosphorus sensitive plants
Total phosphorus	% dry mass	≤ 0.1 for phosphorous sensitive plants
Ammonium	mg/L	< 300
Ammonium + nitrate	mg/L	≥ 100 if an input to plant nutrition is required
Nitrogen	% dry matter	≥ 0.8 if an input to plant nutrition is required
Organic matter content	% dry matter	≥ 25
Boron	mg/kg	< 200
Sodium	% dry mass	< 1
Moisture content	%	25– 40 (maximum dependent on % organic matter)

During the process of composting, compost goes through a predictable series of biological, physical and chemical processes. A schematic generalisation of the stages in composting is demonstrated in Fig. 3 (adapted from Epstein 1997). In the first stage, known as the primary mesophilic stage of composting, the fresh compost contains readily degradable compounds that can be utilised by mesophilic microorganisms resulting in a rapid increase in temperature from ambient temperature to around 60°C. As the process continues, temperature stabilises at approximately 55-70°C, humic substances and thermophilic bacterial numbers increase, while human, plant and animal pathogens, weed seeds and other microorganisms are destroyed.. In the final stage of composting, known as the secondary mesophilic stage, the temperature falls gradually from 55-70°C to ambient temperature and remains at this temperature for several weeks. In the early secondary

mesophilic phase, mesophilic microorganisms, which are often different from the primary mesophilic microorganisms, recolonise the compost including those with the potential to suppress plant disease, such as actinobacteria, *Trichoderma, Ulocladium, Penicillium* and *Cladosporium* (Ryckeboer *et al.* 2003b). In the late secondary mesophilic phase, there is a reduction in the amount of degradable products available and consequently a decline in microbial activity (Hoitink and Boehm 1999, Tuomela *et al.* 2000, Ryckeboer *et al.* 2003a).

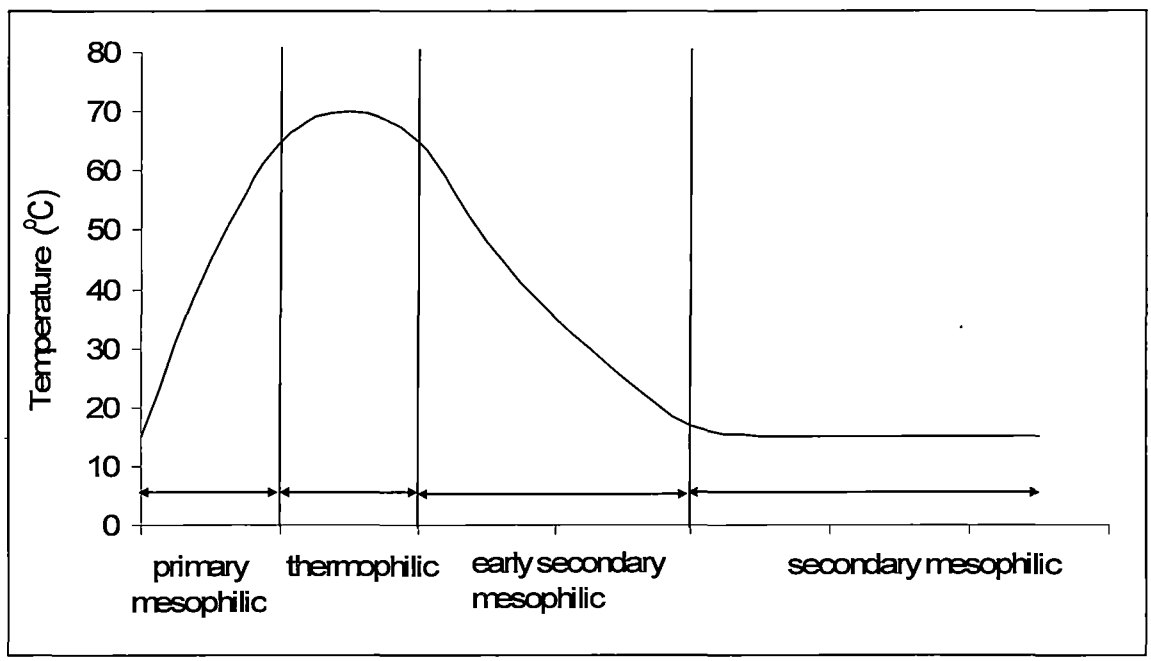


Figure 3 Schematic, generalised representation of the temporal variation in compost internal windrow temperature during aerobic composting.

To date, there has been an extensive range of compost types used for production of ACE. These have included waste products of animal manure, green and woody plant material or a combination of several waste products. Waste ingredients should be combined to obtain an initial C:N ratio of 25-30:1 for rapid composting leading to a finished product with a C:N ratio of 10:1 (Shilesky and Maniotis 1969, Golueke 1992, Toumela *et al.* 2000). Microorganisms use carbon as an energy source and nitrogen for growth. A high concentration of nitrogen in the initial waste ingredients can lead to volatile ammonia (Pagans *et al.* 2006) and unpleasant odours (Finstein

and Morris 1975). Furthermore, excess nitrogen is leached into the environment and can contaminate riparian zones leading to algal blooms and reduced water quality. The U.S. Environmental Protection Agency (Anon. 1998) has produced evidence of contaminated water, dead fish, reduced biodiversity, odours and toxic organisms near composting plants and farms, most likely due to nitrogen compounds from these producers. In contrast, a high concentration of carbon can lead to reduced microbial activity and slower degradation of compost and a deficiency in nitrogen necessary for plant growth when compost is applied to crops (Boulter *et al.* 2000). During composting the balance between carbon and nitrogen should be closely monitored, examined and controlled. It is also important that researchers investigating ACE state the C:N ratio of the initial and final compost because the ratio is an important determinant of the metabolic pathways of composting (de Bertoldi *et al.* 1996). The C:N ratio might also be an important predictor of the antimicrobial activity of ACE, but this hypothesis needs to tested empirically.

Water source and oxygen

The water used for ACE production should be dechlorinated. Chlorine in ACE may destroy or inhibit the activity of the microorganisms with a likely decline in the efficacy of the extract (Ketterer 1990).

There appears to be consensus among scientists and practitioners that ACE should be made with adequate levels of dissolved oxygen for continual activity and reproduction of aerobic microorganisms (Lasaridi and Stentiford 1998, Al-Dahmani *et al.* 2003, Table 4). Anecdotal reports suggest that maintenance of adequate oxygen levels is necessary otherwise non-aerobic conditions may lead to the production of numerous acids including butyric, propionic and acetic acids (Merrill and McKeon

1998, Bess 2000). These acids and many alcohols produced by non-aerobic organisms may be potentially phytotoxic. Non-aerobic conditions have also been suggested to stimulate reproduction of human pathogenic fungi (Ingham 2000). There is no evidence to support these claims and indeed out of approximately 30 published articles on N-ACE none have reported symptoms of phytotoxicity following application of N-ACE to the fruit and foliage of crops.

Table 4 Method of aeration employed for production of aerobic compost extract

Method of aeration	Reference
Submerged pump for circulating water around a porous bag holding compost	Ingham (1999)
A hose which flows water over compost on a screen suspended above a barrel of the resultant extract containing a stirrer.	Riggil (1996)

Salinity

The electrical conductivity (EC) of ACE should be tested prior to application to the crop canopy so that the risk of phytotoxicity due to excess salt is minimised. The salts that are generally found in compost include potassium chloride, sodium chloride, various nitrates, compounds involving sulfates, and calcium, magnesium, and potassium carbonates (Watson 2004). The EC of compost is determined by the initial ingredients and the compost maturity. Sources of raw materials that are rich in nutrients, such as animal manure, will lead to compost with higher EC levels than those not containing animal manure (Watson 2004). Early secondary mesophilic compost has a much higher conductivity than late secondary mesophilic compost and the maturation process reduces the level of conductivity through leaching of salts and/or through microbial osmotic activity (Lau and Wong 2001). Issues of high conductivity might arise in ACE when nutrient amendments are included during

extraction. Ideally, the EC of compost should be as low as the level tolerated by the target crop plant. The effect of EC on soil has been well described, whereas the effect of EC in materials applied to fruit and foliage of crops does not appear to have been reported explicitly.

Table 5 describes different ranges of EC in soil and the influence of EC on crop yield, noting that each crop and plant species has a different susceptibility to a particular salinity level. The upper limit of conductivity at which grapevines can grow without negative impact is 4.7 dS/m in the soil (Qian *et al.* 2004). Different cultivars have variable tolerances for salinity in soil. For instance, Tee *et al.* (2004) reported that cultivars Shiraz, Pinot Noir, Cabernet Sauvignon and Merlot, grown on 'own' roots, were sensitive to high levels of salt in the river basin soil of the Murray-Darling region in Australia, whereas rootstock cultivars Paulsen, Ruggeri and Schwarzmann grown in the same region were salt tolerant.

Table 5 Effect of electrical conductivity (EC) in soil on crop health, using a 1:2 volumetric test (Anon. 1999).

Salinity level in the soil	Electrical conductivity (dS/m)	Crop response
Non-saline	< 1	Negligible
Very slightly saline	1–2	yield reduction in sensitive crops
Slightly saline	2–3	yield of most crops, except tolerant crops, affected
Moderately saline	3–6	yield of tolerant crops unaffected
Strongly saline	> 6	yield of only very tolerant crops unaffected

pH

The optimum pH of finished compost should be between 5 and 7.5, depending on compost use (AS 4454 2003). At this pH level, the compost is neither highly acidic nor does it contain a high level of ammonia. Neutral pH is appropriate for most applications of ACE to soil or the crop canopy. The pH of ACE should be comparable to the compost, except when pH-modifying nutrients are introduced to ACE. For example, if citric acid is added to ACE as a food source for microorganisms, it will reduce the pH. The possible consequence is that the growth of fungi is enhanced because the optimum pH for growth of most bacterial species is between 6.5 and 8, while for fungi, optimum pH is between 2 and 6.5 (Matthies *et al.* 1997).

Extraction time and compost to water ratio

It has been suggested that the efficacy of ACE depends on the duration of extraction (Scheuerell 2003). According to Ingham (1999), the time of extraction in aerobic systems should be 18–24 h, because after this period non-aerobic organisms may begin to multiply. However, there is no scientific evidence supporting this statement. In theory, aerobic conditions should be maintained in ACE when microorganisms are supplied with sufficient oxygen to maintain growth and metabolism. An extended duration of extraction, nevertheless, may lead to a circumstance where all nutrients present in ACE are utilised by the aerobic microorganisms and there is a decline in microbial activity and diversity. As long as aerobic conditions are maintained, it is postulated that the microbial qualities of ACE will be determined primarily by the initial compost source and microorganisms colonising compost from the external environment.

The quantity of compost in water also presumably influences biological and chemical characters of ACE and potential to control plant pathogens. Studies on compost to water ratio in relation to pathogen suppression are limited to N-ACEs. This means that the information cannot be applied directly to ACE because production conditions, compost source and experimental conditions vary. However, these studies illustrate how the effectiveness of extracts contrast with different compost to water ratios and provide a guide as to what ratios may be tested in experiments with ACE. According to Weltzien (1990), N-ACE controlled the plant pathogen *Phytophthora infestans* on detached potato leaflets when the compost to water ratio ranged between 1:3 and 1:10 but not when the compost to water ratio was 1:50. A more detailed *in vitro* experiment by Cronin *et al.* (1996) with *Venturia inaequalis,* the cause of apple scab or black spot, determined the EC_{50} (the concentration that inhibited germination of 50% of *V. inaequalis* conidia) of non-aerobic slurries of compost. There was a linear relationship between the $\log_{10}$cfu (colony forming units) of the extract concentration and germination inhibition. The extracts were mixed with sterile deionised water in ratios ranging from 1:3.16 to 1:100. In contrast to studies of Weltzien (1990) and Cronin *et al.* (1996), the quantity of compost in water did not influence grey mould suppression on field grown strawberries (Welke 2004). In conclusion, a standardised ACE should be developed by testing a range of well-characterised composts, extraction times and compost to water ratios.

Additives

Nutrient availability is critical for microbial metabolism and growth (Eqli and Zinn 2003). Nutrients added to ACE might increase the activity and abundance of microorganisms. However, some materials rich in protein and amino acids, like fish emulsion or fish hydrolysate, may enhance microbial reproduction (El-Tarabily *et al.*

2003) to a degree where oxygen is consumed faster than it is introduced by water circulation or addition of oxygen gas, leading to non-aerobic conditions. Nutrients might also alter microbial composition. Ingham (2000) suggested that bacterial-dominant or fungal-dominant ACEs can be prepared by selecting appropriate nutrient amendments but presented no scientific evidence in support of these claims.

The benefit of using ACE amended with nutrients appears to depend on the pathosystem. Travis *et al.* (2003) compared ACE with ACE modified by addition of humic acids, fish hydrolysate and granular molasses and found no significant difference between treatments in the suppression of powdery mildew and botrytis bunch rot on glasshouse-grown grapevines (Travis *et al.* 2003). In another study, the most consistent ACE for suppression of damping-off on field-grown cucumbers caused by *Pythium ultimum* was ACE amended with kelp, humic acids and rock dust (Scheuerell and Mahaffee 2004). In another pathosystem, Scheuerell and Mahaffee (2006) illustrated no significant difference between unamended ACE and ACE with nutrients for grey mould suppression on geranium, regardless of an increase in microbial numbers with additional nutrients (Scheuerell and Mahaffee 2006). Further research is necessary to elucidate whether microbial numbers, as influenced by additives or other factors, have a positive effect on pathogen inhibition and disease suppression.

Many ACE practitioners test new recipes regularly with the goal of achieving enhanced plant production, soil structure and nutrient cycling in the soil, plus reduced disease incidence or severity (Ingham 2000). These recipes appear to be adopted from recommendations made by salespeople without any controlled scientific experimentation to support their use. Presently, the additives that are sold

commercially for use with ACE include molasses, sugar, brewer's yeast, seaweed, rock dust, fish hydrolysate, fish emulsion and calcium carbonate. If ACE can be standardised for consistent suppression of a particular pathogen, then calcium carbonate is a potential additive for enhanced effectiveness of ACE. Calcium carbonate lowers the pH of a solution (McLean *et al.* 1961) and may increase the abundance of fungal species, given that the optimum pH for fungi is between 2 and 6.5 (Matthies *et al.* 1997). If the additive does make the ACE 'fungal dominant', then this treatment could be used to test the hypothesis that fungal dominant ACEs are more effective than other ACEs.

Storage and application

Survival of microorganisms under storage

The survival time for aerobic microorganisms after extraction and before application has not yet been studied scientifically. Foregoing commercialisation of bio-fungicides, experiments are required to determine the long-term survival or integrity of the active component/s. Abadias *et al.* (2001) discovered that the greatest survival rate and viability of *Candida sake* occurred when the biocontrol agent was freeze-dried and stored in lactose and skim milk. In contrast, Sandoval-Coronado *et al.* (2001) illustrated successful storage of aerial dried *Pseudomonas fumosoroseus* at 4°C for 45 days. While these types of studies indicate the potential for biocontrol agents, including ACE, to have a 'shelf life', more research is required to determine the viability of microorganisms in ACE during storage and transport. The ability to store ACE will depend on the rate of oxygen use by microorganisms, the capacity to moderate microbial metabolism, the survival rate of microorganisms under storage, the potential to concentrate ACE produced in large quantities and the possibility of microbial competition, parasitism and antibiosis within ACE. If ACE is to be

developed commercially, and if its shelf life is short, then it is likely to be produced and sold only at a regional and/or local level.

Modifying ACE prior to application

Adjuvants are mixed with synthetic fungicides to reduce the surface tension of the spray droplets, helping the fungicide to adhere and increase its surface area on the leaf or fruit surface (Zabkiewicz 2007). By this mechanism and their chemical nature, adjuvants improve wetting, wax solubilisation and active transport of the fungicide across the leaf (Steurbaut 1993). Spray adjuvants have been shown to increase the effectiveness of ACEs in reducing *B. cinerea* development on glasshouse grown geranium (Scheuerell and Mahaffee 2006). In grapevine, however, a number of adjuvants have been shown to disintegrate the epicuticular waxes on berry surfaces, causing greater susceptibility to *B. cinerea* infection and depletion of the number of microorganisms on the fructosphere (Marois *et al.* 1987, Rogiers *et al.* 2005). The addition of organic adjuvants to ACE needs to be investigated further to determine if adjuvants reduce the number of microorganisms and consequently the effectiveness of ACE. Fish hydrolysate and fish emulsion contain fish oil and could be tested as potential organic adjuvants (de Ong 1927).

Application

Prior to application of ACE, assessment of the spray equipment will be essential to ensure survival of the microorganisms as they pass through pumps at high pressure and through spray nozzle orifices. The type of sprayer, nozzle and pump will influence the velocity, pressure, droplet size and coverage of ACE on the crop canopy. The mechanism of action of ACE will determine the spray interval and timing in relation to pathogen infection: either before the disease is established

(protective spray) or after disease is established (eradicant spray). Finally, the interval between spraying events will depend on crop phenology and susceptibility, disease pressure and the survival of microorganisms from ACE in changing environments.

Microbial abundance and dynamics in the phyllosphere

Information on the dynamics and composition of microorganisms on the surface of plant tissue is essential to gain a greater understanding of the changes that occur when microorganisms in ACE are added to the phyllosphere. Changes in microbial ecology can then be correlated to the level of disease suppression. In addition to glasshouse and detached tissue assays, it is important that phyllosphere microbiology is studied within the field environment, because changing environmental conditions (temperature, humidity, light and moisture) will alter the dynamics of microorganisms on the host surface.

Only one study has evaluated microbial composition on grapevine leaves following application of compost extract. Sackenheim *et al.* (1994) applied a combination of horse manure N-ACE, brewer's yeast and sucrose to grapevine leaves and discovered that the bacterial and fungal species which inhabited the leaf surface depended on the nutrients added to N-ACE and the foliar microclimate. In this study, the abundance of microorganisms was determined by spreading various dilutions of ACE on a solid medium required for microbial growth. This technique is a useful tool to measure the relative abundance of culturable microorganisms but it may not detect a large proportion of taxa that are non-culturable (Amann *et al.* 1995). Since the study of Sackenheim *et al.* (1994), DNA techniques have been developed to determine the

abundance of both culturable and non-culturable microorganisms (Garland *et al.* 2001).

Two DNA techniques, single stranded conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE), can be used to study microbial communities as described by Theron and Cloete (2000) and Liu *et al.* (2002). SSCP represents conformational changes of single-stranded RNAs/DNAs in solution and has been used to determine the succession and diversity of microbial communities during composting (Peters *et al.* 2000), while DGGE has been used to investigate the effect of addition of organic fertiliser amendments to soil on bacterial community succession (Marschner *et al.* 2002). Individual DNA bands generated by DGGE or SSCP that are associated uniquely with a particular sample or group of samples can be cloned and sequenced. These techniques might be useful to investigate changes in ACE microbial populations in the phyllosphere over time.

In comparison with DGGE and SSCP, terminal restriction fragment length polymorphism (T-RFLP) appears to be a more reproducible molecular technique for routine analysis of microbial communities. T-RFLPs are generated by PCR with fluorescently labelled primers that are digested with restriction enzymes to produce DNA fragments of different lengths (Osborn *et al.* 2000). These fragments are separated by gel or capillary electrophoresis and each fragment is called a terminal restriction fragment (T-RF) because each fragment is considered to represent a single microbial species or a taxon comprising closely-related species (Hill *et al.* 2003). The advantage of T-RFLP over DGGE and SSCP is that T-RFLP appears to resolve a greater diversity of microorganisms and multiple samples can be loaded simultaneously (Liesack and Dunfield 2002, Nunan *et al.* 2005). Both DGGE and T-

RFLP have been used to analyse bacterial communities in submerged rice plants and have generated similar results; however, T-RFLP detected a greater diversity of bacteria than DGGE (Horz *et al.* 2001). Unlike DGGE and SSCP, gel analysis is rapid and the computer output can be compared to sequence databases, such as the T-RFLP fragment sorter Fragsort 5.0 (Michel and Sciarini 2003). The disadvantage of T-RFLP over DGGE and SSCP is that each T-RF could represent a number of microbial species. However, application of multiple restriction enzymes can adequately determine if a sequence is present or absent from the community and allows comparison of microbial diversity for microbial communities.

A greater understanding of microbial survival and the dynamics of microbial communities on grapevine leaves and berries in relation to the application of ACE, using culture-based and DNA techniques, will aid production and application of ACE for maximum suppression of foliar and fruit diseases.

Mechanism of disease suppression

Many practitioners claim disease suppression following application of ACE to crops, but to date there is no clear explanation for the mechanism or mechanisms involved in pathogen and thus disease suppression (Sackenheim *et al.* 1994, Elad and Shtienberg 1994, Merrill and McKeon 1998, Scheuerell 2003). McQuilken *et al.* (1994) provided evidence that the abundance of microorganisms determined pathogen suppression by comparing filter-sterilised and heat-sterilised N-ACEs to non-sterilised extracts and their ability to suppress *B. cinerea* infection on bean leaflets. Weltzien (1989), who studied downy and powdery mildew on grapes, also found no effect of filter or heat-sterilised N-ACEs on spore germination or disease development indicating that it was activity of microorganisms that was associated

with pathogen and disease suppression. In contrast, Cronin *et al.* (1996) reported that metabolites of non-aerobic microorganisms contributed to pathogen suppression. Similarly, Al-Dahmani *et al.* (2003) reported a heat stable metabolite produced in compost prepared with cow manure, pine bark or yard waste that was correlated to disease suppression of bacterial spot on tomato. However, this metabolite was inconsistently produced in these composts. Consistent pathogen and disease suppression appears to be achieved with extracts containing metabolically active microorganisms (Ketterer 1990, Stindt and Weltzien 1990, Urban and Tränkner 1993). The only peer reviewed paper investigating filter sterilisation of ACE demonstrated that living microorganisms were essential for disease control (Scheuerell and Mahaffee 2004).

Possible explanations for disease suppression with ACE include those that explain the various mechanisms of single agents for biological control (Elmer and Reglinski 2006). These mechanisms include induced plant resistance to disease, competition with the plant pathogen for nutrients, hyperparasitism and direct inhibition of the target plant pathogen by production of antimicrobial compounds including chitinolytic enzymes. Mechanism of action must be understood in order to develop a reliable recommendation for the use of a new biological control agent and this knowledge will help target research towards increased efficacy and consistency in results. If ACE production was standardised and its composition predictable, then it might become a candidate for registration as a crop protectant. Information on mechanism of action would be necessary for the registration process.

Nutrients in the infection court are essential for *B. cinerea* conidial germination and host infection (Holz *et al.* 2004). Therefore, microorganisms present in ACE might

suppress *B. cinerea* spore germination by competing for nutrients. Germination of *B. cinerea* conidia has been inhibited by isolates of *Rhodotorula glutinis* and *Cryptococcus albidus* colonising beet root leaves, bean and tomato plants (Elad 1996). *R. glutinis* and *C. albidus* were able to outcompete *B. cinerea* and suppress germination over a wide range of nutrient levels. Several of the single biological control agents that can outcompete *B. cinerea* may be present in ACE. The level of disease suppression accomplished by competition is dependent on biotic (eg. plant defence responses) and abiotic factors such as temperature, relative humidity, and nutritional environment (Guetsky *et al.* 2002). Future studies on the mechanism of action of ACE should be performed over a wide range of temperature, humidity and nutritional environments. The challenge in such studies is that fluctuating environmental conditions found in the field are difficult to simulate in controlled-environment incubators.

Antibiosis is evident when there is lysis of hyphal cell walls of a pathogen without physical contact between the pathogen and the antagonist. Antagonistic microorganisms degrade cell walls of adjacent pathogen hyphae by releasing volatile and non-volatile antibiotics (Lederer *et al.* 1992). El-Masry *et al.* (2002) produced an ACE *in vitro* from composted green waste in which no evidence of antibiotics or siderophores was detected; however, there were lysogenic enzymes present that may have accounted for some of the inhibitory activities of ACE. In contrast, Dianez *et al.* (2006) evaluated the presence of siderophores in ACE made from grape marc compost and illustrated that siderophores were partially responsible for the *in vitro* suppression of nine soil-borne pathogens. Suppression was suggested to be due to a combination of factors, including competition, lysogenic enzymes and antibiotics, although further experimental evidence is required.

Weltzien and Ketterer (1986) were the first to suggest induced resistance in grapevine due to application of N-ACE. A type of induced resistance, known as systemic acquired resistance (SAR), is a broad-spectrum resistance, which develops systemically in the plant after it is attacked by a pathogen (Ross 1961). SAR is activated by a chemical elicitor that initiates a complex set of reactions and ultimately results in the development of host defence responses against the plant pathogen (Zhang *et al.* 1998). There have been several types of elicitors recognised (Guetsky *et al.* 2002). Synthetic analogues of natural chemical elicitors such as benzo (1,2,3) thiadiazole-7-carbothioic acid (S) methyl ester and probenazole have been commercialised for crop protection and sold as Bion® and Oryzemate®, respectively (Görlach *et al.* 1996, Sekizawa and Mase 1980).

SAR can also be induced by environmental microorganisms (Kessmann *et al.* 1994). For example, *Pseudomonas fluorescens,* present naturally in soil, induces resistance in tobacco leaf tissue (Kessmann *et al.* 1994). Induced resistance by naturally occurring microorganisms is an exciting new prospect for biological control of plant pathogens. Some organisms in ACE may secrete chemical elicitors and induce plant resistance and others may have direct antagonistic effects against pathogens. In order to demonstrate induced resistance, it is necessary to rule out direct impact of ACE on each stage of the pathogen life cycle and develop or apply an assay that detects induced resistance using a biochemical or molecular marker associated with the host-pathogen system under study.

Human pathogens

The principal safety concern surrounding ACE is the potential for survival, re-establishment and growth of human pathogens during production and/or application. Human pathogens have been found in both aerobic and non-aerobic compost extracts when nutrients have been added to the extracts (Kannangara *et al.* 2006, Ingram and Millner 2007). Potential sources of human pathogens in ACE are from the compost source, contaminated water used during extraction and/or direct contamination from workers handling equipment without appropriate microbial safety protocols employed.

If human pathogens are introduced to ACE they will proliferate if the growth conditions are suitable. Levels of oxygen, pH, temperature, salinity, and duration of extraction, additives and the field environment will determine the survival of these organisms. To prevent the risk of human pathogen regrowth, Lung *et al.* (2001) recommended that compost used for extraction should maintain at least 45°C for 72 h during composting and that additives should not be used during extraction. There is still a risk of human pathogen contamination after the thermophilic phase of composting because some bacteria such as *Bacillus cereus* can survive as spores during the thermophilic stage of composting (Bollen 1993). Other human pathogens might contaminate the compost from environmental sources, for example, *Listeria monocytogenes.* Sidorenko *et al.* (2006) showed that high temperature soils containing an abundance of humic and fulvic acids stimulate reproduction of *L. monocytogenes.* Compost is high in humic and fulvic acids and *L. monocytogenes* is an environmental bacterium that might be able to compete well in compost.

Scheuerell and Mahaffee (2002) suggested that addition of nutrients, especially molasses, during ACE production, is the primary reason for re-establishment and growth of human pathogens. At least three studies involving additives to ACE report establishment and regrowth of human pathogens in ACE. Duffy *et al.* (2004) inoculated ACE with *E. coli* O157 H7 and a *Salmonella* sp. and found that addition of molasses at the beginning of ACE extraction led to an increase in colony counts of these human pathogens. Regrowth of these bacteria did not occur below 0.5% molasses, but was observed when 0.5 or 1% of molasses was added. The laboratory-scale methods used by Duffy *et al.* (2004) to make ACE were not representative of current extraction techniques used commercially. Furthermore, only one cfu of *E. coli* per ml was added to each extract, thus increasing the potential for an extended lag phase and mortality of the bacterium during inoculation. In addition, oxygen, pH and conductivity were not measured throughout the extraction process. In contrast, Kannangara *et al.* (2006) and Ingram and Millner (2007) prepared ACEs in the laboratory which resembled commercial ACE production and *E. coli* was inoculated in sufficient numbers, ranging from 1×10^1 to 1×10^7 cfu/ml across both studies. Both research groups measured the oxygen level and pH during extraction and maintained a consistently high level of oxygen during ACE production. In both studies, numbers of *E. coli* were below the limit of detection in non-inoculated extracts, with or without additives, and there was no increase in *E. coli* numbers in inoculated, unamended extracts.

According to Kannangara *et al.* (2006), when powdered kelp or molasses were introduced to N-ACE and ACE, there was a significantly higher number of *E. coli* in N-ACE. There was however, a linear relationship between the concentration of molasses or kelp and the number of *E. coli* in ACEs, and the number of *E. coli* in

ACE amended with powdered kelp was lower than observed in ACE amended with molasses. Interestingly, carrot juice reduced the number of *E. coli* in ACE and increased the number of microorganisms. Kannangara *et al.* (2006) used carrot juice because carrot cells are thought to secrete phytoalexin 6-methoxymellein, an antimicrobial substance.

Unlike the results of the study by Kannangara *et al.* (2006), Ingram and Millner (2007) found that there was a significantly greater number of *E. coli* and *Salmonella* sp. in ACE than N-ACE, when commercially available bacterial supplements, kelp, humic acid or rock dust were included in the extracts. *E. coli* are facultative anaerobes that have been shown to proliferate in both aerobic and non-aerobic conditions (Unden *et al.* 1994). The dissimilar human pathogen growth patterns in ACE and N-ACE in these experiments may be due to variation in nutrient type, nutrient concentration, pH, conductivity or compost source, rather than oxygen level. Further research should compare ACE amended with nutrients to the equivalent concentration of nutrients in water (without compost extraction) as well as the survival of human pathogens at various pHs, salt levels and nutrient concentrations.

To safeguard agricultural workers and/or prevent the food supply from becoming contaminated, the ecology of human pathogens in the cropping environment where ACE has been applied, including microclimates that favour their regrowth, should be investigated.

Summary

There is an increasing trend world-wide for greater adoption of sustainable agricultural practices. ACE appears to be a promising alternative to synthetic

fungicides that could be integrated into crop disease management. Research on N-ACE dates back to the 1980s, and when compared to ACE, there is more experimental evidence for its effectiveness *in vitro*, and *in planta* under controlled and field conditions. ACE, with its short production time, has been adopted widely by practitioners in recent years. This review of the literature demonstrates that ACE has the potential to suppress certain pathogens and diseases. The lack of demonstrated effectiveness of ACE in field grown crops to date indicates that ACE needs to be developed further and standardised for maximum and consistent pathogen suppression. Understanding the mechanism of action of ACE and the potential for human pathogen regrowth will inform production techniques and patterns of application for maximum safety and control of foliar and fruit diseases. Detailed studies of microbial ecology of the phyllosphere should also be performed to understand the impact of ACE application in relation to plant pathogen development in various cropping environments.

PROJECT AIMS

The primary aim of this research was to standardise the production of aerobic compost extract (ACE), using compost produced by a collaborative industry partner, Soil First Pty Ltd, so that each batch produced consistently high levels of suppression of two major fruit and foliar diseases of grapevine (*Vitis vinifera* L.). *Botrytis cinerea* and *Erysiphe necator*, the cause of botrytis bunch rot and powdery mildew, respectively, were used to identify and standardise an ACE that would consistently reduce infection and colonisation of these two plant pathogens with different life history and biology.

The specific objectives of this research were:

1) To define qualities of compost and an extraction process that produced an extract that suppressed the growth and reproduction of *B. cinerea* significantly and consistently *in vitro.*
2) To investigate the mechanism of powdery mildew suppression in relation to the time of infection of glasshouse grown grapevines by conidia of *E. necator.*
3) To assess the effectiveness of ACE in reducing powdery mildew and botrytis bunch rot when applied to disease susceptible varieties of wine grapes grown in commercial vineyards.
4) To determine the survival of microorganisms in the phyllosphere after treatment of leaves with ACE in the field (commercial wine grape production).
5) To investigate the safety of ACE for commercial production and application:
    - To determine the presence of human pathogens in compost and ACE.

- To assess the capacity of human pathogens to grow in ACE and ACE amended with nutrients.

CHAPTER 1:

STANDARDISED PRODUCTION OF AEROBIC COMPOST EXTRACT (ACE) FOR SUPPRESSION OF THE COSMOPOLITAN PLANT PATHOGEN, *BOTRYTIS CINEREA*

1.1 Abstract

Aerobic compost extract (ACE) is a watery extract of aerobic compost that favours the persistence of aerobic microorganisms. ACE is applied to the soil or to the fruit and foliage of horticultural crops to improve plant health but there is limited scientific evidence to support its use for crop protection. Three composts with variable raw ingredients were used to evaluate production variables for ACE systematically. A reproducible bioassay was used to assess the impact of different ACEs on the growth and reproduction of the fungal pathogen *Botrytis cinerea* on detached bean leaflets. Bacterial-dominant ACEs produced with a compost weight to water volume ratio of 1:3 to 1:30, and from compost sampled in the early secondary mesophilic stage of composting inhibited *B. cinerea* colonisation of bean leaflets to a greater extent than ACEs produced from compost sampled in later mesophilic stages. There was limited evidence to suggest that the magnitude of pathogen suppression was associated positively to the number of bacterial and fungal Terminal Restriction Fragments (T-RFs) or microbial taxon diversity in ACE. ACE directly inhibited the germination of *B. cinerea* conidia *in vitro* and removal of microorganisms from ACE by filtration reduced but did not eliminate its capacity to inhibit conidial germination. Water-soluble antibiotics were not detected in filtered ACE following an *in vitro* assay for the inhibition of *B. cinerea* colony growth. Standardised production of ACE, including sampling the compost windrow at an internal temperature of 40–

50°C, can now be adopted for evaluating ACE for plant disease management in the field.

1.2 Introduction

B. cinerea is a cosmopolitan and necrotrophic fungal pathogen that infects a wide range of fruit, vegetable and ornamental crops (Coley-Smith *et al.* 1980). Diseases of flowers and fruit caused by *B. cinerea*, including bunch rot of wine and table grapes and grey mould of strawberries, can lead to significant loss of crop yield and quality (Sutton 1998, Elad *et al.* 2004). Many horticultural producers worldwide use multiple applications of synthetic fungicides to protect crops from infection by *B. cinerea.* There is significant market and legislative pressure to reduce fungicide inputs because of concerns about the development of pathogen resistance to fungicides and the safety of fungicides to humans and the environment. Integrating alternative control measures, such as cultural and biological controls, is the first step to reducing fungicide inputs while maintaining cost-effective disease management. Biological control has focussed on the use of one or several well-characterised fungal or bacterial species to target a specific plant pathogen. Potential mechanisms of action of biological control agents include the production of antibiotic compounds (Cronin *et al.* 1996, Haggag and Saber 2007), cell wall degrading enzymes (Elad 1994, Metcalf 2002), siderophores (Leong 1986, Dianez *et al.* 2006), competition for nutrients in the pathogen infection court (Buck 2002), and/or the induction of the defence response in the plant host (Elad 2000, Magnin-Robert *et al.* 2007). The approach to biological control investigated in this study, application of aerobic compost extract (ACE), is a type of multiple-target biological control that has been poorly characterised.

Growing numbers of farmers, farm service providers, landscape gardeners and golf course managers are producing and applying ACE to soil or plant canopies for improved plant health and putative suppression of a wide range of plant pathogens (Touart 2000, Scheuerell and Mahaffee 2002). ACE is a watery extract of compost that is produced aerobically using extraction conditions that favour growth of aerobic microorganisms from the original compost. It is produced within 72 h, in custom-built extraction tanks, and applied to the soil or to fruit and foliage soon after production. Practitioners report the amendment of ACE with nutrients such as kelp extract, rock dust, humic acid or molasses during extraction in order to promote microbial abundance and disease suppression (Ingham and Alms 1999, Ingham 2003). There are many anecdotal reports that application of ACE reduces the incidence and severity of a range of fungal and bacterial plant diseases (eg. Diver 2002, Ingham 2003), yet few scientific studies that support these claims. Inhibition of the germination of pathogen spores by ACE *in vitro* has been reported, for example, ACE prepared from spent mushroom compost reduced the germination percentage of conidia of *Venturia inaequalis,* the cause of apple scab (Cronin *et al.* 1996).

Apart from *in vitro* studies, there appears to be only one scientific, peer-reviewed report that demonstrates that application of unamended ACE can reduce the incidence of disease relative to water or non-treated controls. During a natural epidemic of botrytis grey rot on field grown strawberries, Welke (2004) illustrated a significant reduction in disease incidence when berries were treated with bi-weekly application of unamended ACE in comparison with berries treated with water or non-treated berries. In a recent study, Scheuerell and Mahaffee (2006) evaluated the effectiveness of different ACEs prepared from various compost substrates, with or without adjuvants or additives, by applying treatments to leaves of glasshouse grown

geraniums prior to inoculation with *B. cinerea*. Only ACEs amended with kelp extract, rock dust, humic acid or adjuvants such as yucca extracts or spreader/stickers reduced the severity of grey mould relative to the non-treated control, but these results were inconsistent given that significant reduction occurred in just seven of 27 experiments. Only one study has used commercially available ACE applied *in vitro*, *in vivo* and in field trials on potato crops for control of the late blight pathogen *Phytophthora infestans* (Sturtz *et al.* 2006). This commercial product did not appear to reduce the growth of *P. infestans* or the incidence of late blight significantly.

The multitude of production techniques for ACE (Scheuerell and Mahaffee 2002) and lack of a standardised method of ACE production among studies has made it difficult to evaluate the effectiveness of ACE for consistent crop protection. Furthermore, the capacity to standardise production of an ACE that suppresses disease consistently has not been fully explored. Systematic evaluation of production parameters for ACE starts with the desired carbon to nitrogen (C:N) ratio at compost windrow initiation (Shilesky and Maniotis 1969, Tuomela *et al.* 2000), followed by development of criteria for sampling the compost at an appropriate stage during the composting cycle. The presence of microorganisms in compost and hence extracts, has been demonstrated to be a contributing factor to the level of disease or pathogen suppression observed (Scheuerell and Mahaffee 2004, 2006). The number of culturable microorganisms, however, does not appear to be related to the level of pathogen suppression (Scheuerell and Mahaffee 2004, 2006). The abundance, diversity and/or metabolic activity of both culturable and non-culturable microorganisms in ACE needs to be investigated for the potential of this information to indicate which part of the composting cycle produces a disease suppressive ACE.

The primary aim of this study was to develop a systematic process for identifying potentially disease suppressive ACEs prior to evaluating their effectiveness under glasshouse and field conditions. A bean leaflet assay was used to investigate the effect of compost age, internal windrow temperature, extraction time and other production parameters on the ability of different ACEs to inhibit the growth and reproduction of *B. cinerea*. From the ACEs assessed, a number were selected that varied in their capacity to inhibit *B. cinerea* on bean leaflets and were characterised for the abundance of culturable microorganisms and diversity of microorganisms by analysis of community DNA for Terminal Restriction Fragment Length Polymorphisms (T-RFLPs: Liu *et al.* 1997, Clement *et al.* 1998, Osborn *et al.* 2000). The level of pathogen suppression and microbial characters of ACE were then related to the physical and chemical characters of compost from which they were prepared. A second objective was to conduct a preliminary investigation of the mechanism of action of a pathogen-suppressive ACE identified during the initial screening process.

1.3 Materials and Methods

1.3.1 Production of compost

Compost was produced by Soil First Pty Ltd on a commercial scale using open windrow composting (Epstein 1997) at Parrata in central Tasmania, Australia. The compost windrows were in the form ofa long hill ridge measuring 3 m wide and 2 m high. The dominant raw materials, which varied depending on supply of waste products, and production times for specific batches of compost are summarised in Table 1.1. Composting was initiated with a carbon to nitrogen ratio (C:N) of 30:1, based on the recommendations of Shilesky and Maniotis (1969) and Tuomela *et al.* (2000). A hydrolysate of raw salmon waste was added 2 to 3 weeks after

establishment of the compost windrow, which increased the internal temperature of the windrow and decreased the C:N ratio. This practice, while not ideal for producing good quality compost, was based on a commercial need to manage excess salmon waste. In compost windrow two (Table 1.1), raw salmon waste was added a second time at 5 weeks, resulting in an elongation of the thermophilic phase. Compost began to cool from thermophilic to mesophilic conditions approximately 5 to 6 weeks after windrow establishment.

Internal windrow temperature was measured daily at three positions: approximately 10 m from each end of the 50 m windrow and in the centre of the windrow. The temperature probe, a Wavetek Meterman^{TM} Test Instruments Model TM Thermometer with TP254 Immersion Probe, was inserted at a depth of 1 m from the apex of the windrow. Windrow moistness was evaluated daily by a visual technique referred to in the compost industry as the 'squeeze' method (Rynk 1992). Compost was turned weekly with a tractor drawn windrow turner and if necessary water was applied during turning. Electrical conductivity, pH, nitrate, nitrite, ammonium and percentage humus were generally measured when the compost was mature according to AS 4454 for compost, mulches and conditioners (2003), typically 12 to 13 weeks post-windrow establishment.

Table 1.1 The dominant raw ingredients and time to maturity (production time) of compost windrows sampled for preparation of ACE.

Compost windrow	Dominant raw ingredients	Compost production time and seasons during production
one andtwo	cow manure from feedlot beef, *Eucalyptus* spp. sawdust, newsprint waste-water sludge based on plantation *Pinus radiata* and regrowth *Eucalyptus* spp., hydrolysate of raw salmon (*Salmo salar*) from aquaculture filleting waste	87 days, summer/autumn (one) or 76 days, winter/spring (two)
three	chicken manure from organic chicken farms, mulched green waste from municipal councils, hydrolysate of raw cultured salmon (*S. salar*) from filleting waste	79 days, summer/autumn
four	mulched green waste from municipal councils, hydrolysate of raw cultured salmon (*S. salar*) from filleting waste	85 days, winter/spring

1.3.2 Compost extraction method and characters of ACE

Three 100 L tanks were placed in a dark room maintained at 22°C ± 4°C. Each tank contained 30 L of tap water and a pond pump (Bianco, BIAWFP1500, White International Pty Ltd, Milperra, Australia) that circulated the water in a fountain motion at 1,500 L h^{-1} for 24 h for dechlorination (Lawson 1994) prior to compost extraction. A 10 kg sample of compost was collected from each of the three positions within the windrow where internal temperature had been measured, placed in a 20 L container and transferred by road transport of 1 h duration to New Town Research Laboratories for immediate extraction. Each compost sample was placed into a 100% polypropylene sack (40 cm × 70 cm pillow protector) and suspended in one of the three tanks containing dechlorinated tap water. A compost to water ratio of 1:3 was

chosen to replicate ACE production conditions employed by the Soil First Pty Ltd. The pond pump continued to circulate the water for maintenance of aerobic conditions for microbial metabolism. The duration of compost extraction was varied by sampling extract from each tank at specified times after extraction commenced. The pH, dissolved oxygen, conductivity, temperature and nitrate were measured immediately prior to extraction and every 24 h, up to 72 h. Nitrate was determined from nitrate Merckoquant® strip tests (Merck Pty Ltd). The other physical parameters were measured using WTW Handheld 340i or Inolab meters (Merck Pty Ltd).

1.3.3 Bean leaflet assay

Detached bean leaflets inoculated with *B. cinerea* conidia were used to quantify the growth and sporulation of *B. cinerea* in response to treatment of bean leaflets with a particular ACE prior to inoculation. The bean leaflet assay was based on a method reported by Bouhassan *et al.* (2004). *Vicia faba* L. (South Australian Faba bean breeding program, clone 1142), large seeded broad beans, were grown from seed in a glasshouse at 22°C ± 4°C. Seedlings were transferred individually to 20 cm-diameter pots containing premium potting mix (75% composted bark, 20% sand, 5% peat fibre including dolomite lime, gypsum, Osmoform® and Osmocote®) and watered daily. A single leaflet was collected from the third node of each of four bean plants per experimental treatment. Leaflets were surface sterilised by washing in sterile distilled water before transfer to 0.5 g L^{-1} sodium hypochlorite for 3 min and finally rinsed three times in sterile distilled water. The leaflets were then placed on sterile paper towel and dried in a laminar flow hood. Each leaflet was immersed in 4 ml of a specified compost extract for 30 s and placed individually in 90 mm-diameter Petri

plates lined with sterile paper towel moistened with 2 ml of sterile water. The leaflet was allowed to dry before inoculation with a suspension of *B. cinerea* conidia.

Strain DAR 78113 of *B. cinerea* was isolated in May 2003 from naturally infected berries of *Vitis vinifera* variety Chardonnay grown commercially in the Coal River Valley of southern Tasmania. This isolate was maintained on slopes of Pectin Agar (PA) (Metcalf 1997) at 4°C for the duration of experimentation. Pectin agar contained the following (g/L): $NH_4H_2PO_4$, 0.9g; $(NH_4)_2HPO_4$, 2.0 g; $MgSO_47H_2O$, 0.1 g; KCl, 0.5 g; and citrus pectin, 10.0 g (Sigma Aldrich Co., NSW, Australia). The pH was adjusted to 4.0 before addition of 30 g of agar and autoclaving. Conidia of *B. cinerea* strain DAR 78113 were generated from 14-day old cultures on PA in Petri plates that had been maintained in darkness (Stewart and Long 1987) at 21°C ± 4°C. These cultures were scraped from the Petri plate into sterile deionised water, shaken vigorously and the mixture filtered through two layers of cheesecloth to remove agar and mycelial fragments. The suspension was adjusted to 1×10^5 *B. cinerea* conidia ml^{-1} with the aid of a haemocytometer. Six 10 µl drops of suspension were then placed on the adaxial surface of each surface sterilised bean leaflet, with three drops on each side of the midrib. Plates were then sealed with Parafilm™ and incubated in darkness at 21°C ± 4°C. After 48 h, the number of necrotic lesions per leaflet was scored and after 5 days the area of necrosis per leaflet (mm^2) was measured by photography and image analysis using the UTHSCSA ImageTool for Windows, Version 3 program (available from ftp://maxrad6.uthscsa.edu). After 9 days, the number of necrotic lesions with sporulating *B. cinerea* per leaflet was recorded.

1.3.4 Effect of compost age and duration of extraction on the capacity of ACE to inhibit *B. cinerea*

The bean leaflet assay was used to quantify the effect of compost age and duration of extraction on suppression of growth and sporulation of *B. cinerea* by ACE. The experiment was conducted for compost windrows one, two and three listed in Table 1.1 and comprised four compost extraction times (0, 24, 48 and 72 h) and up to nine compost ages, based on weekly sampling of each compost windrow and windrow position from approximately 55°C (cooling phase) until compost maturity. At 0, 24, 48 and 72 h from the onset of extraction, 100 ml of each extract was collected from each tank, representing each windrow position, and maintained at 4°C for no longer than 4 days. The 0 h extraction time represents the tank water prior to addition of compost. Samples of ACE were brought to room temperature prior to treatment of bean leaflets. The abundance of culturable bacteria and fungi, or colony forming units (cfu)/ml, was estimated for each extract by serial dilution, whereby 100 µl of each dilution was spread evenly on each of four plates of nutrient agar (NA) or potato dextrose agar acidified to pH 3.5 with lactic acid (APDA), respectively. Colonies on the NA plates were predominantly bacteria; those that did not represent a presumptive bacterium (shiny appearance, discrete colonies and distinct margins) were not counted. Conversely, colonies on the APDA plates were predominantly fungi; those that did not represent a presumptive fungus (fuzzy appearance) were not counted. Plates of NA were inverted and incubated at 25°C for 72 h and plates of APDA were incubated at 21°C ± 4°C for 7 days prior to colony counts.

1.3.5 Effect of compost to water ratio during extraction on the capacity of ACE to inhibit *B. cinerea*

The bean leaflet assay was used to determine the minimum compost to water ratio required for maximum suppression of growth and sporulation of *B. cinerea*. Compost four (Table 1.1), with an internal temperature of 50°C in the cooling phase, was sampled from the centre of the windrow approximately 1 m from the apex for preparation of five batches of ACE in separate 100 L tanks. Each tank contained different compost to (dechlorinated) water ratios. Using a total water volume of 30 L, each tank contained 30, 10, 3, 1 or 0.3 kg of compost corresponding to a 1:1, 1:3, 1:10, 1:30 or a 1:100 compost weight to water volume ratio. Compost extract samples were collected from each tank at 0, 24, 48 or 72 h after extraction had commenced and 100 ml of each extract was used to treat bean leaflets. The abundance of culturable bacteria and fungi (log_{10}cfu/ml) for each extraction time was determined. This experiment was performed three times using the same bulk sample of compost.

1.3.6 Diversity of microorganisms in selected ACEs

The diversity of microorganisms in selected extracts prepared from compost windrow three (Table 1.1) was determined by analysis of Terminal Restriction Fragment Length Polymorphisms (T-RFLPs: Liu *et al.* 1997, Clement *et al.* 1998, Osborn *et al.* 2000). Extracts analysed were 48-h extracts prepared from compost sampled at the central position in the windrow (tank 2) from the 'production variables' experiment (section 1.3.4) and for each compost age. Three 1 ml samples of each ACE were concentrated by centrifugation at 13,000 × g (Hettick Zentrifugen Mikro 20) at room temperature for 15 min. The cell pellet was resuspended in 1 ml of resuspension buffer (50 mM Tris, 150 mM NaCl, 50 mM EDTA, pH 8). Total

community DNA was extracted from each 1 ml sample of ACE using the Fast Prep DNA kit for Soil (MP Biomedicals, NSW, Australia). The isolated DNA was purified further using a Qiaquick PCR purification kit (Qiagen, Victoria, Australia) and quantified by gel electrophoresis in 1% agarose buffered by 1 × TAE.

Bacterial 16S rDNA genes from total community DNA were amplified by universal eubacterial primers (Osborne *et al.* 2006): 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Intergenic spacer regions of fungal rDNA genes (White *et al.* 1990) were amplified with ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). In all PCR reactions the forward primer was labelled with WellRED dye D3 and the reverse primer was labelled with WellRED dye D4 (Sigma-Proligo, NSW, Australia). Bacterial and fungal PCR was carried out in a 50 µl volume containing 20 ng of template DNA, 20 pmol of each primer, 1 × OptiBuffer (Bioline, NSW, Australia), 1.5 mM $MgCl_2$, 10 mM each of dATP, dCTP, dGTP and dTTP (dNTP mixture, Bioline, NSW, Australia) and 4 U of BIO-X-ACT Short DNA polymerase (Bioline, NSW, Australia). Using an Eppendorf Mastercycler, reactions consisted of: (i) one cycle of 5 min at 95°C (ii) 25 cycles of 30 s at 95°C, 30 s at 55°C, 1 min at 70°C and (iii) a final extension step of 10 min at 70°C. Two PCRs using the same DNA sample were performed. The duplicate PCRs were pooled using the Qiaquick PCR purification kit (Qiagen, Victoria, Australia) and 5 µl of PCR products were visualised by electrophoresis using 1.5% Agarose in 1 × TAE buffer.

Fluorescently labelled PCR products (100 ng) were digested with 5 U of restriction enzymes *Hae*III, *Hha*I or *Msp*I (New England Biolabs Inc., Queensland, Australia) in a 20-µl reaction volume. All reactions were incubated at 37°C for 3 h and

deactivated by heating for 20 min at 65°C (*Hha*I and *Msp*I) or 80°C (*Hae*III). The digested PCR products were desalted using a Qiaquick Nucleotide Removal Kit (Qiagen, Victoria, Australia) and stored at -20°C until digested, desalted PCR products were separated by capillary electrophoresis (Frag-4 default setting, CEQ 8000, Beckman Coulter automated sequencer, NSW, Australia). Each sample (5 µl) was added to 30 µl of sample loading solution and 0.25 µl of a 600 bp molecular ruler size standard comprising 32 different length fragments from 70 to 640 nucleotides (Beckman Coulter Inc., NSW, Australia) labelled with dye D1 (Beckman Coulter Inc, NSW, Australia). Run conditions were a capillary temperature of 50°C, sample denaturation for 120 s at 90°C, sample injection for 30 s at 2 kV and a separation time of 60 min at 4.8 kV.

Bacterial and fungal T-RFLP data were analysed separately. The fragment length and peak height of the terminal restriction fragments (T-RFs) were determined by comparison to the 600 bp size standard using the Beckman Coulter CEQ 8000 fragment analysis software, algorithm v. 2.1.3. A separate profile was produced for each of the three restriction-enzyme digests. When at least two of the triplicate ACE samples produced the same T-RF then this T-RF was considered accurate. Each T-RF was scored as present or absent and analysed as binary data. Terminal restriction fragments with peak heights of less than 100 florescence units were excluded from analysis. The number of T-RFs generated depends on the restriction enzymes used and a single T-RF can represent several species or taxa. Therefore, data from the profiles for the three restriction enzymes were combined for statistical analyses. The number of T-RFs for each sample of ACE was denoted S_{bac} for bacterial richness or S_{fungi} for fungal richness. The total number of T-RFs observed across the three samples of ACE analysed per compost age was denoted total S_{bac} or total S_{fungi} and

the total number of T-RFs observed across all compost ages was denoted 'grand total S_{bac}' or 'grand total S_{fungi}'. The proportion of T-RFs for each sample of ACE was then defined as the S_{bac} or S_{fungi} divided by 'grand total S_{bac}' or 'grand total S_{fungi}'.

1.3.7 Mechanism of action

1.3.7.1 *Effect of ACE on germination of B. cinerea conidia*

An *in vitro* assay was conducted to determine whether or not ACE or modified ACE directly inhibited germination of *B. cinerea* conidia. ACE was prepared from compost four (Table 1.1) with an internal windrow temperature of 50°C. ACE was modified after 24 h of extraction, by addition of one or two nutrients. Foundation Fish™ (FF, raw salmon waste, Table 1.1) was added at a concentration of 1 part FF: 120 parts ACE (v/v). Liquid Kelp™ (LK), comprising Bull Kelp (*Durvillaea potatorum*), was added at a concentration of 1 part LK: 60 parts ACE (v/v). The nutrient concentrations were equivalent to the concentrations used by Soil First Pty Ltd for commercial production of ACE. After 48 h extraction, 100 ml of ACE or modified ACE was collected in a sterile container. Microorganisms were removed from 25 ml of ACE or modified ACE by passing it through a sterile 0.2 µm filter (Schleicher and Schull). Unfiltered or filtered ACE or modified ACE was diluted with sterile dechlorinated water by adding 25, 15, 5, 1.67 or 0.5 µl extract in a total volume of 50 µl, corresponding to extract:water ratios of 1:1, 1:3, 1:10, 1:30 or 1:100, according to Cronin *et al.* (1996). In summary, the treatments were a sterile dechlorinated water control and variable dilutions of unfiltered or filtered ACE, ACE modified with FF, ACE modified with LK or ACE modified with FF and LK.

Each treatment (n = 9), in a 50 µl volume, was mixed with 50 µl of 1×10^5 *B. cinerea* conidia ml^{-1} sterile water in a well of a 96-well ELISA plate. The conidial

suspension was prepared as described previously, except that 0.05 % (v/v) Tween 20 was added to the sterile deionised water to dislodge conidia from PA plates. Treatments were randomised within blocks of four replicates in two ELISA plates. The plates were covered with Parafilm™ and left in darkness at 21°C ± 4°C for 24 h. After 24 h, each 100 µl solution was spread evenly on deionised water agar in a 90 mm-diameter Petri plate. The plates were incubated in darkness at 21°C ± 4°C and after 24 h, 100 conidia per plate were evaluated for germination. Conidia were considered germinated if the hypha was at least the length of the conidium and the number of germinating conidia was expressed as a proportion.

1.3.7.2 ***Assay for the presence of water-soluble antibiotics in ACE*** The potential for water-soluble antibiotics produced by microorganisms in ACE to inhibit the growth of *B. cinerea in vitro* was investigated using ACE or modified ACEs prepared for the germination assay described above. After 48 h extraction, microorganisms were removed from 100 ml of ACE or modified ACE by filtering through a sterile 0.2 µm filter (Schleicher and Schull).

An antibiotic assay was developed with slight modifications to the well-cut diffusion technique described by El-Masry *et al.* (2002). Conidia of *B. cinerea* were added to Mueller-Hinton agar (Oxoid, Australia Limited) at 45°C, to a final concentration of 1 × 10^5 conidia ml^{-1} of agar. Ten 90 mm-diameter Petri plates were prepared, each containing 15 ml of inoculated agar. Six wells were cut out of the agar, using an 11 mm-diameter sterile cork borer, approximately 1 cm from the edge of the Petri plate and two drops of sterile water agar were added to the base of each well. Into each well, 100 µl of one of six treatments was added: filtered ACE; filtered ACE with LK; filtered ACE with FF; filtered ACE with FF and LK; sterile distilled water or sterile

distilled water containing 10 μg μl^{-1} tetracycline hydrochloride (Sigma-Aldrich Co., USA). Each treatment was placed in a random order in each Petri plate and ten Petri plates were prepared in this manner. The plates were sealed with Parafilm™ and placed in the dark. After 7 days, the area surrounding the well where the growth of *B. cinerea* was inhibited was measured using UTHSCSA Image Tool for Windows, Version 3. The edges of the *B. cinerea* colonies near the treatment wells were also observed using a stereomicroscope at 400 × magnification for signs of abnormal hyphal growth.

1.3.8 Data analyses

General Analysis of Variance (ANOVA) in Genstat® for Windows, 8th or 9th Edition, was used to compare treatment means as indicated in the tables of results along with the residual degrees of freedom (df) and the least significant difference (lsd). If there were no significant differences among means for variables measured at each extraction time for a given compost age, then the data for different extraction times were combined for ANOVA of the effect of compost age.

For bean leaflet assays, the mean number of lesions (sporulating or not) per leaflet and area of necrosis per leaflet of the four replicate bean leaflets was subject to principal component analysis (PCA, Jolliffe 2002). The first principal component (PCA1) was defined as the negative average of the number of lesions (sporulating or not) and the area of necrosis associated with pathogen colonisation. The second principal component was defined as a contrast between number of lesions and area of necrosis plus the number of sporulating lesions. PCA1 eigenvector values were used to generate means for ANOVA. In short, the higher the PCA1 eigenvector value, the greater the level of *B. cinerea* inhibition on bean leaflets and a negative eigenvector

value can be interpreted as large areas of necrosis and profusely sporulating lesions on bean leaflets. For the experiment investigating compost age and duration of extraction, compost samples from each windrow position and hence compost extraction tanks represented blocks. Simple linear regression was also performed to identify significant correlations ($P < 0.05$) between mean PCA1 and internal windrow temperature, mean abundance of culturable bacteria or fungi, or mean proportion of bacterial or fungal T-RFs (microbial diversity).

Bacterial and fungal T-RFLP data were also analysed using multi-dimensional scaling (MDS) of Euclidean distances fitted using monotonic regression (non-metric MDS) in Genstat® for Windows 8th Edition. The degree of correspondence between distances in the MDS plot was measured by a stress function, which illustrated the relationship between the MDS points and the actual distances between treatments. A stress value less than 0.2 represents meaningful data and a value greater than 0.3 generally represents a poor description of the ordination (Clarke and Warwick 2001).

For the germination assay, the linear relationship between the mean proportion of germinating *B. cinerea* conidia (n = 4) and the dilution of the extract with water was analysed using general linear regression analysis with groups in Genstat® for Windows 9th Edition. Equality of the regression intercepts and slopes between unfiltered, unamended ACE and each of the other ACE treatments was determined by analysis of variance after arcsine transformation (Milligan 1987) of the proportion of germinating conidia.

1.4 Results

1.4.1 Characters of compost and compost extracts The temperature of each compost windrow declined, as expected, in the cooling phase of composting until compost maturity at 12 to 13 weeks after windrow initiation (Fig. 1.1). Compost extracts prepared from compost windrows one, two and three were used for the compost age/duration of extraction experiment and had a mean pH of 7.1–7.7, a mean dissolved oxygen of greater than 6.5 mg/L, a mean conductivity of 6.7–7.6 dS/m and a mean temperature was 23.9–26.6°C (Table 1.2). Nitrate was present in all extracts except those prepared 5 and 6 weeks post windrow initiation (Table 1.2). Nitrate was also present in all extracts prepared using compost four, which was used for the studies of mechanism of action (Table 1.7).

1.4.2 Effect of compost age and duration of extraction on the capacity of ACE to inhibit *B. cinerea*

Compost extract prepared from all three compost windrows and various compost ages inhibited the growth and sporulation of *B. cinerea* on bean leaflets (Fig. 1.2, Tables 1.3, 1.4). The mean PCA1 eigenvector value indicated a significant ($P < 0.001$) effect of each ACE on *B. cinerea* colonisation of bean leaflets relative to the control (0 h extraction time, Table 1.3). There was no significant difference among extraction times for the mean level of pathogen suppression. The mean number of culturable bacteria and fungi in the 24-h extracts, prepared from composts two and three, was slightly but significantly higher when compared with the numbers observed with longer extraction times (Table 1.3).

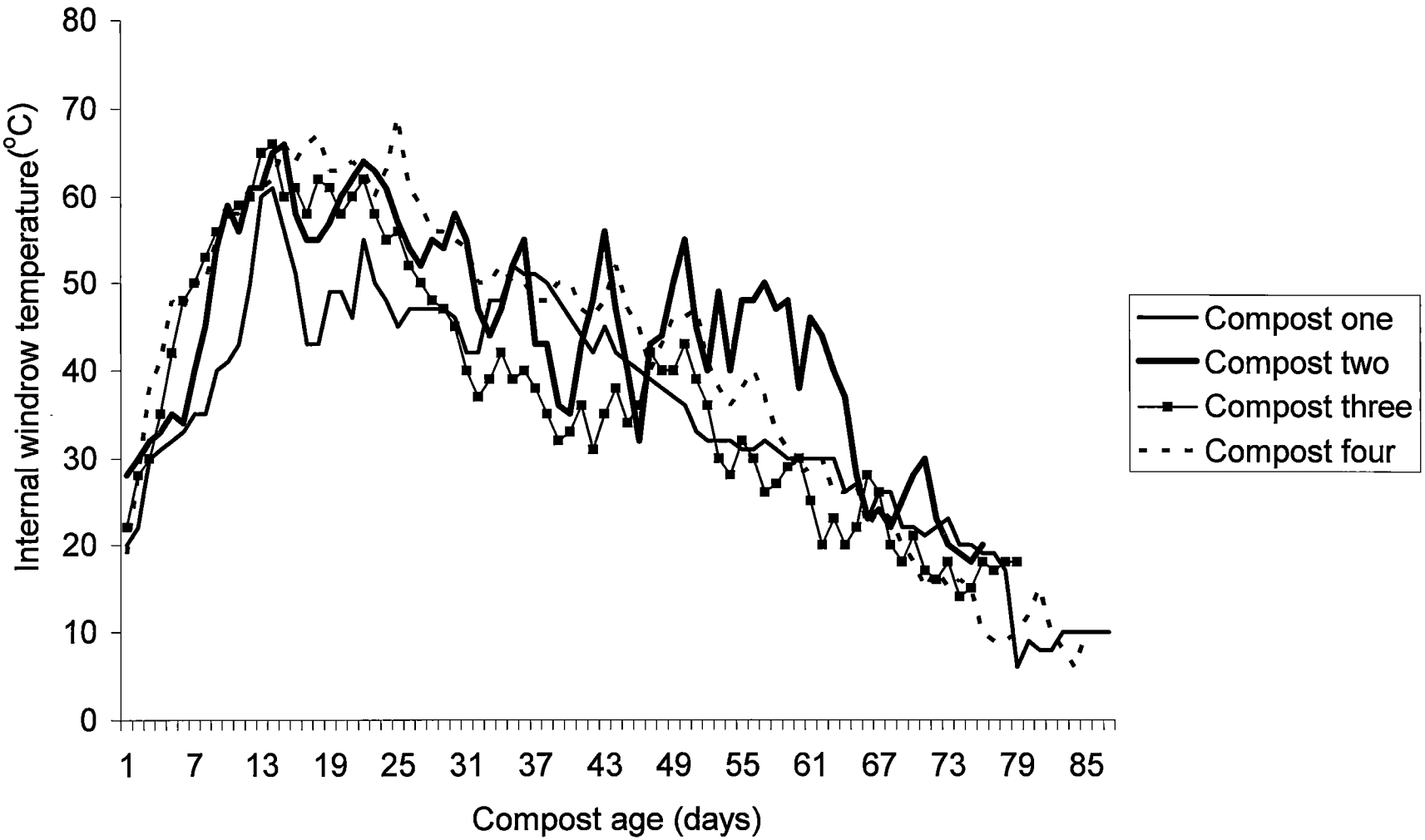


Figure 1.1 Internal windrow temperature for compost windrows one, two, three and four.

Figure 1.2 Bean leaflets immersed for 30 s in 4 ml of A. 48 h ACE prepared from compost with an internal windrow temperature of 50°C and B. 0 h ACE (dechlorinated water), dried and inoculated with six 10 µl drops of 1 x 10^5 *Botrytis cinerea* conidia m^{-1} and incubated at 21°C ± 4°C in darkness for 5 days. Necrotic lesions, symptomatic of infection by *B. cinerea*, developed on inoculated leaves treated with dechlorinated water (B).

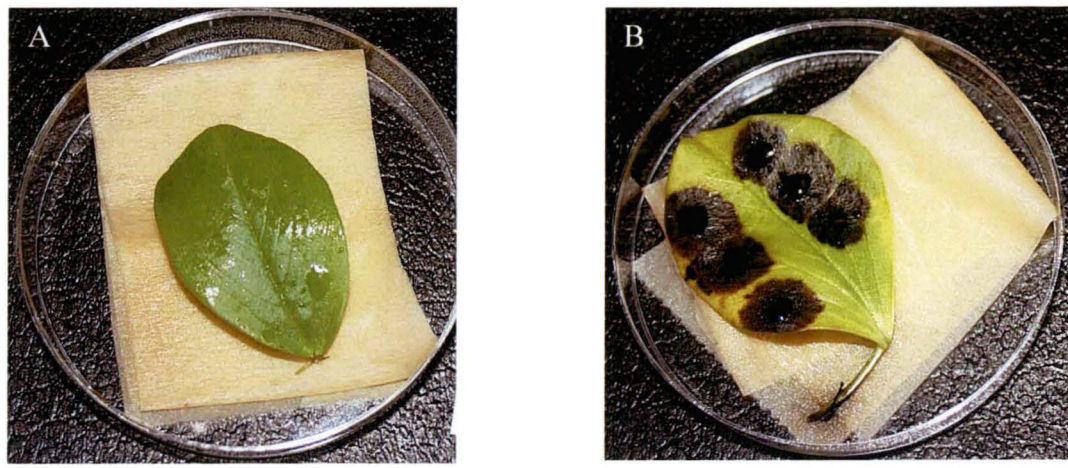


Table 1.2 Mean physical characters of ACE for compost windrows one, two and three. Means (± standard deviations) for compost age were from data combined from extraction times of 24, 48 and 72 h. Means for compost extraction time were from data combined from all compost ages. The 0 h extraction time represents the tank water prior to addition of compost.

Compost age (weeks)	pH	Dissolved oxygen (mg/L)	Conductivity (mS/cm)	Temperature (°C)	Nitrate (mg/L)
5	7.5 ± 0.7	7.0 ± 0.4	7.6 ± 0.7	26.5 ± 3.4	0.0 ± 0.0
6	7.7 ± 0.4	6.7 ± 0.3	7.3 ± 2.7	26.6 ± 3.3	0.0 ± 0.0
7	7.5 ± 0.4	6.6 ± 0.5	6.8 ± 1.5	26.2 ± 3.7	35.8 ± 79.0
8	7.5 ± 0.4	6.8 ± 0.5	6.4 ± 1.6	26.1 ± 3.7	108.2 ± 163.2
9	7.3 ± 0.3	7.0 ± 0.8	6.8 ± 1.5	26.1 ± 1.8	132.5 ± 202.5
10	7.2 ± 0.3	7.4 ± 0.8	7.6 ± 1.4	26.0 ± 1.9	105.0 ± 135.0
11	7.5 ± 0.4	7.0 ± 0.5	7.6 ± 1.5	25.2 ± 1.5	243.3 ± 207.0
12	7.5 ± 0.3	7.0 ± 0.6	7.4 ± 1.7	24.4 ± 0.7	398.3 ± 143.6
13	7.1 ± 0.2	7.6 ± 0.2	6.9 ± 1.7	23.9 ± 0.7	300.0 ± 120.9
Compost extraction time (h)					
0	7.2 ± 0.3	8.1 ± 0.7	1.1 ± 1.4	24.0 ± 2.4	0.0 ± 0.0
24	7.4 ± 0.4	7.2 ± 0.6	6.8 ± 1.8	25.6 ± 2.4	149.5 ± 191.7
48	7.5 ± 0.4	6.9 ± 0.5	7.6 ± 1.4	26.0 ± 2.7	191.8 ± 217.0
72	7.5 ± 0.4	6.9 ± 0.6	7.2 ± 1.5	25.8 ± 2.9	183.6 ± 183.6

The internal compost windrow temperature accounted for 92.2%, 92.5% or 93.3% of the variance in *B. cinerea* inhibition on bean leaflets when PCA with two principal components was calculated for compost windrows one, two and three, respectively. The internal windrow temperature accounted for 62.4% (windrow one), 77.2% (windrow two) and 71.2% (windrow three) of the variance when the first principal component (PCA1) was analysed. ANOVA of PCA1 eigenvector values revealed that the mean PCA1 eigenvector values were relatively high when the internal windrow temperatures were 51°C, 50°C and 48°C for compost windrows one, two and three, respectively (Table 1.5). However, in compost windrow two there was no significant difference in PCA1 eigenvector values between internal windrow temperatures of 50°C at 9 weeks and 37°C at 10 weeks. There were negative PCA1 eigenvector values for some treatments; namely 17°C for compost windrow one, 56°C in compost windrow two and 30°C and 21°C for compost windrow three. There were relatively large areas of necrosis and mean numbers of sporulating lesions when bean leaflets were treated with extracts prepared from compost sampled whilst at these temperatures (Table 1.6), suggesting little or no inhibition of *B. cinerea.*

For ACE prepared from compost windrow two, the highest number of culturable fungi was observed from a 50°C compost and the highest number of culturable bacteria was observed when compost was cooling from 55°C (week 8) to 37°C (week 10) (Table 1.5). Like compost windrow two, the greatest number of culturable fungi was evident from ACE prepared from compost windrow three at 48°C whereas the greatest numbers of culturable bacteria were evident between 48°C (week 5) and 31°C (week 7). Internal windrow temperature in compost three explained 51% of the variation in the mean number of culturable fungi (P = 0.027 for the linear regression).

1.4.3 Effect of compost to water ratio during extraction on the capacity of ACE to inhibit *B. cinerea*

The first principal component (PCA1) explained between 69.5% and 86.9% of the variation in *B. cinerea* inhibition on detached bean leaflets in relation to the compost to water ratio during extraction (Table 1.7). The PCA1 eigenvector values were highest at compost to water ratios of 1:3, 1:10 and 1:30 relative to other ratios tested. At a compost to water ratio of 1:100 the PCA1 eigenvector value was negative and there were significantly more lesions and a significantly greater area of necrosis on bean leaflets than at any other ratio (Table 1.8). The greatest numbers of microorganisms were present in ACE when the compost to water ratio was 1:1. At this ratio, there was less dissolved oxygen and a higher conductivity than at any other ratio of compost to water. As the compost to water ratio decreased, the temperature appeared to decline but the pH remained constant (Table 1.7).

Table 1.3 First principal component results (PCA1 eigenvector values) of the mean effect of compost extraction time across compost ages on the extent of symptoms and sporulation caused by *Botrytis cinerea* on bean leaflets (n = 4) treated with various compost extracts prior to inoculation. The corresponding mean number of bacteria and fungi (log_{10}cfu/ml) is presented for compost windrows two and three. Means within columns followed by the same letter are not significantly different at $P < 0.001$. Refer to Table 1.4 for the arithmetic means of symptoms and incidence of sporulation.

Compost extraction time (h)	Compost windrow one		Compost windrow two				Compost windrow three			
	PCA1[1]		PCA1		No. of bacteria (log_{10}cfu/ml)	No. of fungi (log_{10}cfu/ml)	PCA1		No. of bacteria (log_{10}cfu/ml)	No. of fungi (log_{10}cfu/ml)
0	-1.90	b	-2.12	b	N/A[2]	N/A	-1.91	b	N/A	N/A
24	0.42	a	0.97	a	7.95 a	6.59 a	0.77	a	9.39 a	7.67 a
48	0.66	a	0.69	a	7.07 b	5.41 b	0.64	a	9.32 b	7.10 b
72	0.82	a	0.47	a	7.13 b	5.00 c	0.50	a	8.93 c	6.80 c
lsd	0.44		0.51		0.16	0.17	0.55		0.04	0.07
df	102		90		4	4	90		6	6
P	< 0.001		< 0.001		< 0.001	< 0.001	< 0.001		< 0.001	< 0.001

[1] The higher the PCA1 eigenvector value, the greater the level of pathogen inhibition.
[2] N/A is not applicable.

Table 1.4 The mean effect of compost extraction time across compost ages on the number of necrotic lesions, area of necrosis and number of sporulating lesions caused by *Botrytis cinerea* on bean leaflets (n = 4) treated with each compost extract prior to inoculation.

Compost extraction time (h)	Compost windrow one			Compost windrow two			Compost windrow three		
	Number of lesions[1]	Area of necrosis (mm^2)[2]	Incidence of sporulation[3]	Number of lesions	Area of necrosis (mm^2)	Incidence of sporulation	Number of lesions	Area of necrosis (mm^2)	Incidence of sporulation
0	5.2	37.3	5.1	5.8	46	5.7	5.8	73	5.9
24	2.3	18.3	2.0	2.5	17	1.2	2.9	46	2.0
48	1.9	13.3	1.9	2.7	18	1.9	2.8	44	2.6
72	2.0	10.2	1.5	2.5	25	2.3	3.2	46	2.5

[1] Mean number from six inoculation points per leaflet.

[2] Mean area from six inoculation points per leaflet..

[3] Number of sporulating lesions from six inoculation points per leaflet.

Table 1.5 First principal component results (PCA1 eigenvector values) of the mean effect of compost age across extraction times for 24, 48 and 72 h on the extent of symptoms and sporulation caused by *Botrytis cinerea* on bean leaflets (n = 4) treated with each compost extract prior to inoculation. Compost age is presented as weeks after windrow initiation and as internal windrow temperature during the early secondary mesophilic stage. The corresponding mean number of bacteria and fungi (log_{10}cfu/ml) is presented for compost windrows two and three. Means within columns followed by the same letter are not significantly different at $P = 0.05$.

Compost age (weeks)	Compost windrow one		Compost windrow two				Compost windrow three			
	T (°C)	PCA1[1]	T (°C)	PCA1	No. of bacteria (log_{10}cfu/ml)	No. of fungi (log_{10}cfu/ml)	T (°C)	PCA1	No. of bacteria (log_{10}cfu/ml)	No. of fungi (log_{10}cfu/ml)
5	47	0.74 ab	54	1.04 abc	7.26 c	5.92 bc	48	1.88 a	9.43 c	8.51 a
6	51	1.38 a	55	0.44 cd	7.03 d	5.22 e	37	0.59 bcd	9.82 b	8.16 b
7	45	0.83 ab	56	-0.56 e	7.10 cd	5.18 e	31	0.48 cd	10.00 a	7.58 c
8	36	0.29 bc	55	0.65 bc	7.82 ab	6.34 b	40	1.31 ab	9.04 d	7.13 d
9	32	0.60 bc	50	1.31 a	7.87 a	6.95 a	30	-0.01 de	8.80 f	6.49 e
10	26	0.84 ab	37	1.35 a	7.76 ab	5.48 d	23	1.05 bc	8.85 e	6.39 e
11	21	0.42 bc	30	1.13 ab	7.66 b	5.54 d	21	-0.59 e	8.86 e	6.13 f
12	17	-0.05 c	20	0.33 d	6.46 e	5.71 cd	18	0.38 cd	8.92 e	7.16 d
13	10	0.66 bc								
lsd		0.71		0.65	0.34	0.53		0.80	0.07	0.17
df		64		56	13	29		56	29	19
P		0.017		< 0.001	< 0.001	< 0.001		< 0.001	< 0.001	< 0.001

[1] The higher the PCA1 eigenvector value, the greater the level of pathogen inhibition.

Table 1.6 The mean effect of compost age across extraction times of 24, 48 and 72 h on the number of necrotic lesions, area of necrosis and number of sporulating lesions caused by *Botrytis cinerea* on bean leaflets (n = 4) treated with each compost extract prior to inoculation. Compost age is presented as weeks after windrow initiation and as internal windrow temperature during the early secondary mesophilic stage.

Compost age (weeks)	Compost windrow one				Compost windrow two				Compost windrow three			
	T (°C)	Number of lesions[1]	Area of necrosis (mm^2)[2]	Incidence of sporulation[3]	T (°C)	Number of lesions	Area of necrosis (mm^2)	Incidence of sporulation	T (°C)	Number of lesions	Area of necrosis (mm^2)	Incidence of sporulation
5	47	2.2	4.0	1.7	54	3.1	6.7	1.1	48	1.1	31	1.1
6	51	1.4	1.8	0.2	55	3.0	26	1.7	39	3.0	60	1.7
7	45	1.6	15	1.4	56	2.9	45	3.8	31	3.3	55	2.0
8	36	2.7	21	2.0	55	3.2	21	1.2	40	2.8	19	1.7
9	32	2.2	14	1.9	50	2.3	8.5	0.8	30	2.9	77	2.9
10	26	1.6	6.3	2.0	37	1.6	12	1.3	23	2.0	38	2.4
11	21	1.6	3.4	1.8	30	2.0	12	1.9	21	4.8	55	3.7
12	17	2.9	50	3.0	20	2.6	26	2.6	18	3.6	30	3.14
13	10	2.3	9.7	1.9								

[1] Mean number from six inoculation points per leaflet.

[2] Mean area from six inoculation points per leaflet..

[3] Number of sporulating lesions from six inoculation points per leaflet.

Table 1.7 First principal component results (PCA1 eigenvector values) of the mean effect of changing the compost to water ratio during extraction, across extraction times of 24, 48 and 72 h, on the extent of symptoms and sporulation caused by *Botrytis cinerea* on bean leaflets (n = 4) treated with compost extract prior to inoculation. ACE was prepared from compost four sampled when the internal windrow temperature was 50°C (week 5), in the cooling phase of composting. The corresponding mean number of bacteria and fungi (log_{10}cfu/ml), and mean characters of ACE are presented for each dilution. Means within columns followed by the same letter are not significantly different at $P = 0.05$ and standard deviations (±) for each dilution were from data combined from extraction times of 24, 48 and 72 h.

Ratio of compost to dechlorinated water in a total volume of 30 L water	PCA1[1]		Number of bacteria (log_{10}cfu/ml)		Number of fungi (log_{10}cfu/ml)		pH	Dissolved oxygen (mg/L)	Conductivity (dS/m)	Temperature (°C)	Nitrate (mg/L)
1:1	0.12	bc	10.4	a	7.2	a	6.7 ± 0.0	2.6 ± 1.6	19.3 ± 1.0	27.4 ± 0.3	66.7 ± 28.9
1:3	1.15	a	8.4	b	7.1	b	7.0 ± 0.2	6.4 ± 0.5	8.7 ± 1.5	25.9 ± 0.2	66.7 ± 28.9
1:10	1.48	a	8.4	b	7.1	b	7.1 ± 0.4	6.6 ± 0.3	4.3 ± 0.1	25.3 ± 0.6	66.7 ± 28.9
1:30	0.72	ab	8.1	c	5.7	c	7.4 ± 0.4	8.0 ± 1.6	0.7 ± 0.5	24.4 ± 1.8	50.0 ± 0.0
1:100	-0.36	c	7.2	d	5.8	c	7.0 ± 0.1	9.3 ± 0.9	0.3 ± 0.2	23.8 ± 1.2	50.0 ± 0.0
lsd	0.84		0.08		0.11						
df	32		9		11						
P	< 0.001		< 0.001		< 0.001						

[1] The higher the PCA1 eigenvector value, the greater the level of pathogen inhibition.

Table 1.8 The mean effect of changing the compost to water ratio during extraction, across extraction times of 24, 48 and 72 h, on the number of necrotic lesions, area of necrosis and number of sporulating lesions caused by *Botrytis cinerea* on bean leaflets (n = 4) treated with each compost extract prior to inoculation. ACE was prepared from compost four sampled when the internal windrow temperature was 50°C (week 5), in the cooling phase of composting.

Dilution	Number of lesions[1]	Area of necrosis (mm^2)[2]	Incidence of sporulation[3]
1:1	2.2	35	3.4
1:3	0.9	23	2.1
1:10	1.7	19	3.0
1:30	1.0	12	1.7
1:100	3.1	61	2.7

[1] Mean number from six inoculation points per leaflet.
[2] Mean area from six inoculation points per leaflet..
[3] Number of sporulating lesions from six inoculation points per leaflet.

1.4.4 Diversity of microorganisms in selected ACEs

T-RFLP appeared to be a reproducible technique because two or three terminal restriction fragments (T-RFs) of equivalent length were observed for each T-RF analysed across triplicate DNA preparations from the same batch of ACE. Analysis of community DNA in ACE prepared from compost windrow three revealed a maximum of 102 bacterial T-RFs when combining results of restriction enzymes for each extract (Table 1.9). A considerably lower number of fungal T-RFs, up to 25 T-RFs across restriction enzymes, were observed (Table 1.9). There was a significantly greater proportion of bacterial T-RFs present in ACE prepared from compost three with an internal windrow temperature of 48°C than in the remaining extracts. The smallest proportion of bacterial T-RFs was observed in ACE prepared from compost at weeks 10 to 12 (23°C–18°C) (Table 1.9). In addition, ACE produced from compost with an internal windrow temperature of 48°C contained the greatest proportion of fungal T-RFs (Table 1.9). There was no apparent trend in the proportion of fungal T-RFs as the compost cooled below 48°C.

Figs 1.3 and 1.4 show a schematic representation of the diversity of bacterial and fungal taxa across extracts prepared from various compost ages and corresponding internal windrow temperatures in compost windrow three. For each enzyme assayed, ACEs prepared from the windrow at weeks 10 to 12 had lower diversities of bacterial T-RFs relative to ACE prepared at week 5 (Fig. 1.3). When bacterial DNA was digested with *Hae*III (Fig. 1.3B), there was a greater diversity of bacterial taxa in weeks 5, 6 and 7 than from week 8 until maturity (week 12). Among all extracts, the greatest diversity of fungal taxa was apparent when ACE was prepared from compost with an internal windrow temperature of 48°C (week 5, Fig. 1.4) and at week 9 for restriction enzyme *Hha*I (Fig. 1.4A).

The results of multidimensional scaling (MDS) of bacterial and fungal T-RFs distinguished ACE prepared from 48°C compost from all other ACEs, based on the distance of this T-RF profile from others (Fig. 1.5). However, there was no grouping of internal compost windrow temperatures in relation to bacterial T-RF profiles (Fig. 1.5A). Conversely, there were two clusters of internal windrow temperatures, specifically 18°C, 21°C and 23°C and 31°C, 39°C and 40°C, in relation to fungal T-RF profiles (Fig. 1.5B). Internal windrow temperatures 30°C and 48°C were far apart on this MDS plot, illustrating dissimilarity in T-RF profiles between these windrow temperatures and the remaining windrow temperatures.

Table 1.9 Results of T-RFLP analysis of 48-h ACEs prepared from compost windrow three with data generated using three restriction enzymes. The total number of T-RFs for three samples of ACE for each compost age is denoted as total S_{bac} for bacterial richness and total S_{fungi} for fungal richness. Mean proportion was calculated from the proportion of T-RFs, which is the S_{bac} or S_{fungi} for each ACE sample divided by the grand total S_{bac} or S_{fungi} observed across compost ages.

Compost age (weeks)	T (°C)	Bacteria			Fungi		
		Total S_{bac}	Mean proportion		Total S_{fungi}	Mean proportion	
5	48	102	0.5	a	25	0.64	a
6	39	70	0.34	b	11	0.28	ed
7	31	54	0.27	b	8	0.21	d
8	40	65	0.32	b	9	0.23	e
9	30	71	0.35	b	20	0.51	b
10	23	25	0.12	d	11	0.28	ed
11	21	43	0.21	c	13	0.33	c
12 (maturity)	18	46	0.23	bc	14	0.36	c
lsd			0.06			0.04	
df			14			14	
P			< 0.001			< 0.001	

1.4.5 Mechanism of action

1.4.5.1 *Effect of ACE on germination of B. cinerea conidia*

ACE, ACE with amendments and filtered ACEs inhibited the germination of *B. cinerea* conidia to varying degrees, with simple linear regression demonstrating the effect of various dilutions of each extract with water (Fig. 1.6). In summary, the germination of *B. cinerea* conidia was not inhibited by dechlorinated water, and the mean germination proportion was 0.97 (data not shown). The mean germination proportion was less than 0.1 at all dilutions of unamended ACE and less than 0.01 for ACE diluted 1:1 with water (Fig. 1.6A). There was no relationship between the various dilutions of ACE amended with FF and the mean germination proportion, which was less than 0.01 at all dilutions (Fig. 1.6B). ACE or amended ACE (Figs 1.6A, B, C, D) inhibited germination of *B. cinerea* conidia to a greater extent than

filtered ACE or filtered amended ACE (Figs 1.6E, F, G, H). The slope and intercept of the regression line for ACE amended with LK was not significantly different from the regression coefficients for ACE (Table 1.10). However, the addition of FF to ACE reduced the intercept of the regression line significantly (Table 1.10) indicating that these treatments inhibited the germination of *B. cinerea* to a greater extent than unamended ACE. The intercepts for all filtered extracts were significantly different from unfiltered ACE, indicating that filtration increased the mean germination proportion of *B. cinerea* conidia. Filtered ACEs amended with FF were the only treatments where the decline in the mean germination proportion with increasing concentration was similar to the ACE treatment. Relative to the ACE treatment, there was a steeper decline in the mean germination proportion with increasing concentration in the other filtered ACEs.

1.4.5.2 *Assay for the presence of water-soluble antibiotics in ACE*

The antibiotic tetracycline, the positive control in the antibiosis experiment, was the only treatment that inhibited the growth of *B. cinerea* significantly *in vitro*, when compared with filtered ACE and the dechorinated water control (Table 1.11). A large proportion of hyphal tips at the edge of *B. cinerea* mycelia in the vicinity of filtered ACE appeared to burst and hyphal contents became coagulated (Fig. 1.7), relative to hyphae in the same relative location in the water control.

Table 1.10 Coefficients for the linear regressions of the proportion of germinating *Botrytis cinerea* conidia (arcsine transformed) and various dilutions of amended and/or filtered ACE prepared from compost four. The *P* value represents the probability of equality of the regression intercepts and slopes between unfiltered, unamended ACE and the ACE treatment specified.

Treatment	Intercept Coefficient	*P* value	Slope Coefficient	*P* value
ACE + FF	1.471	0.001	0.261	0.167
ACE + FF + LK	1.456	0.003	0.175	0.085
ACE + LK	1.296	0.669	0.611	0.972
Filtered ACE	0.197	< 0.001	-0.082	< 0.001
Filtered ACE + FF	0.245	< 0.001	1.565	0.402
Filtered ACE + FF + LK	0.520	< 0.001	0.809	0.367
Filtered ACE + LK	0.767	< 0.001	0.825	0.029

Table 1.11 Mean inhibition of the growth of *Botrytis cinerea* surrounding an agar plug (well) containing one of six treatments: filtered ACE, filtered ACE + LK, filtered ACE + FF, filtered ACE + FF + LK, dechlorinated water control and tetracycline. ACEs prepared from compost four. Plates were incubated in darkness for 7 days. Means followed by the same letter were not significantly different at $P = 0.05$.

Treatment	Area surrounding the well (mm^2)	
Filtered ACE	5.0	a
Filtered ACE + LK	4.8	a
Filtered ACE + FF	4.1	a
Filtered ACE + FF + LK	2.1	a
Dechlorinated water control	7.2	a
Tetracycline (10µg/µl)	98.5	b
lsd	14.32	
df	45	
P	< 0.001	

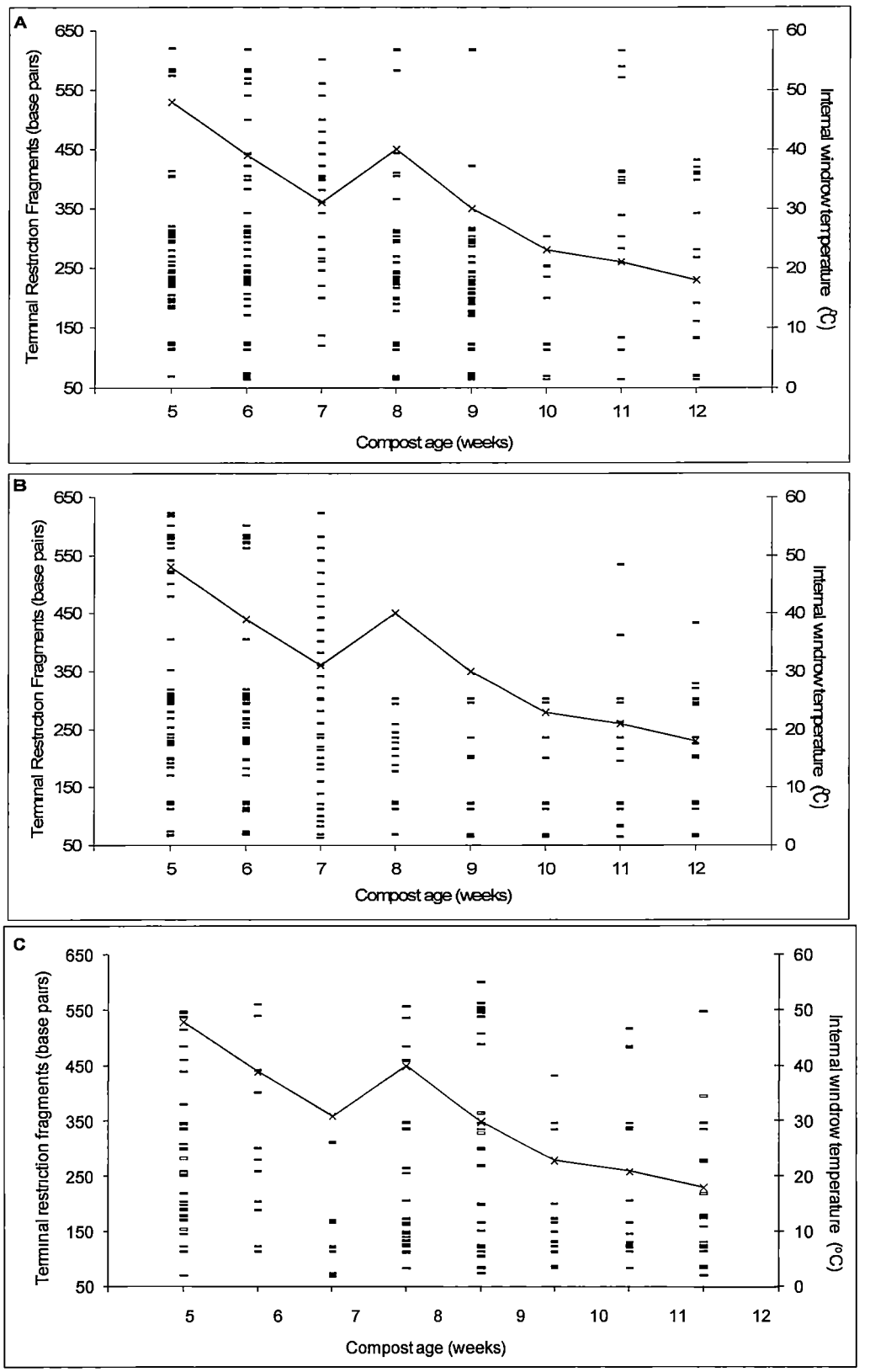


Figure 1.3 Schematic representation of 16S rDNA bacterial T-RFs from community DNA in 48-h ACEs prepared from compost three every week in the cooling phase until compost maturity. T-RF profiles were generated with restriction enzymes *Hha*I (A), *Hae*III (B) or *Msp*I (C). Bars that are wider than the thinnest bar represent more than one T-RF. The solid line represents internal windrow temperature.

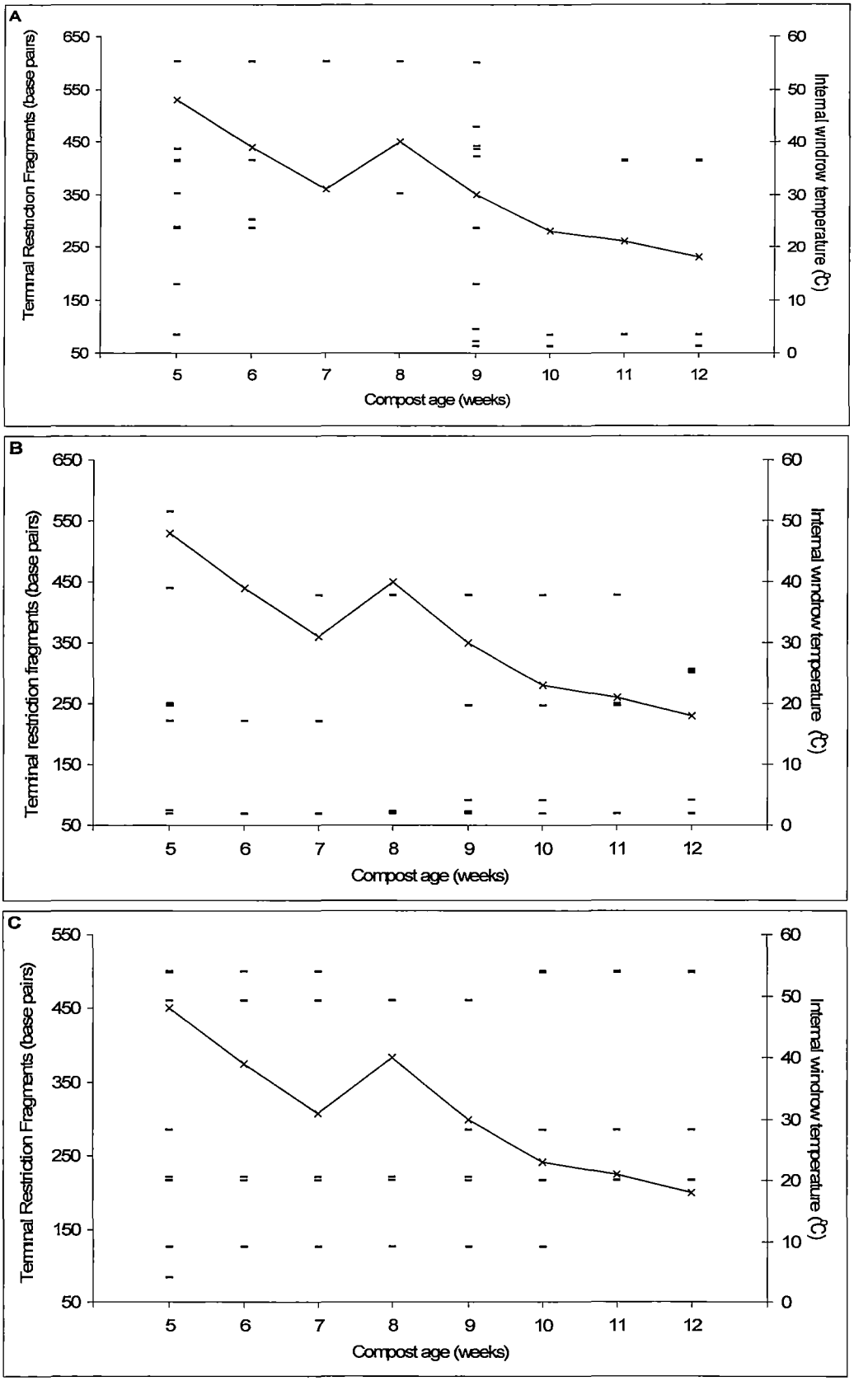


Figure 1.4 Schematic representation of internal transcribed spacer (ITS) fungal T-RFs from community DNA in 48-h ACEs prepared from compost three every week in the cooling phase until compost maturity. T-RF profiles were generated with restriction enzymes *Hha*I (A), *Hae*III (B) or *Msp*I (C). Bars that are wider than the thinnest bar represent more than one T-RF. The solid line represents internal windrow temperature.

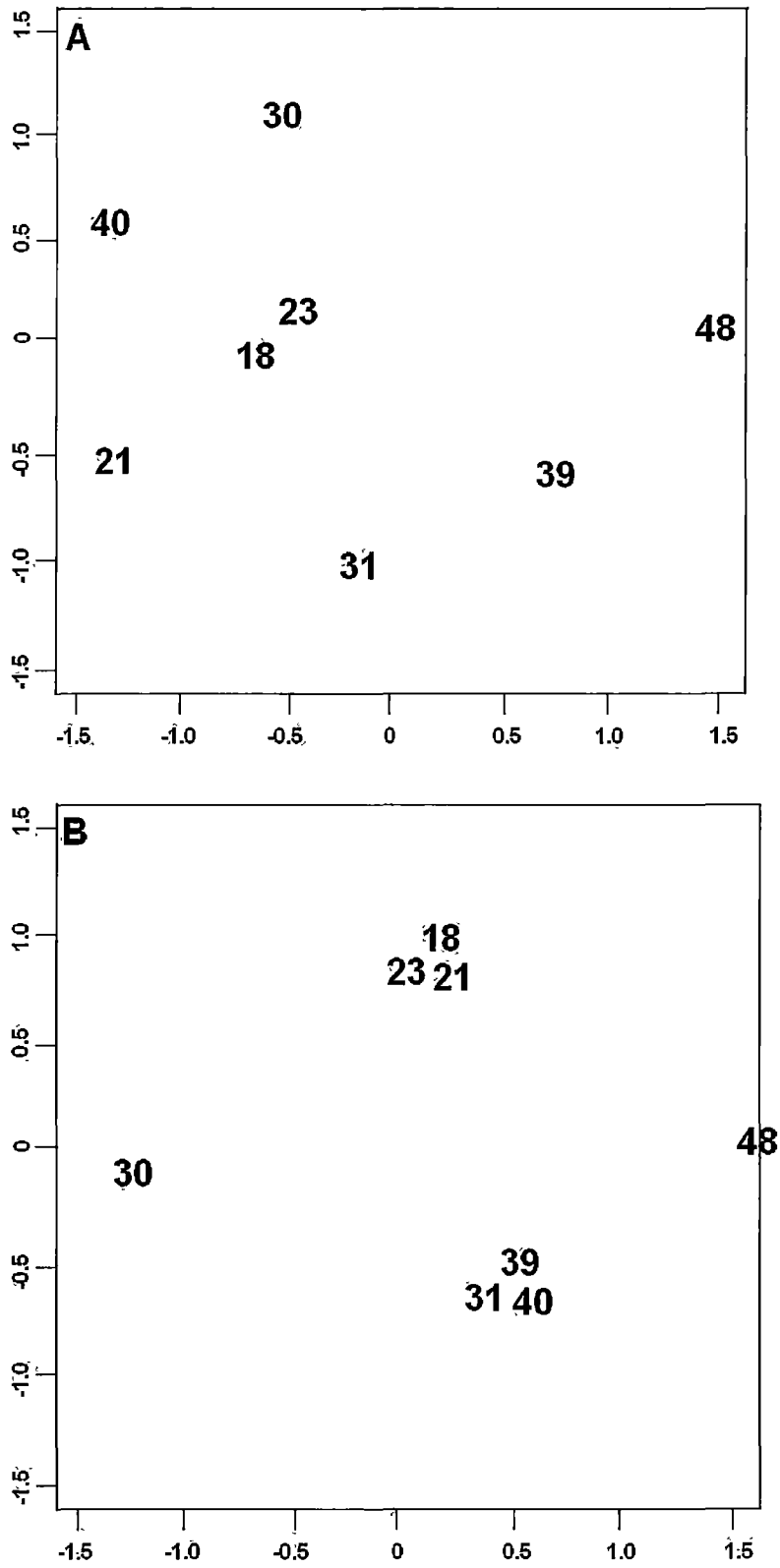


Figure 1.5 Multi dimensional scaling (MDS) of 16S rDNA bacterial T-RFs (A) and internal transcribed spacer (ITS) fungal T-RFs (B) from community DNA in 48-h ACEs prepared weekly from compost three. T-RFs were generated and combined using three restriction enzymes. Numbers between the axes represent the internal windrow temperature. Analyses revealed 2D stress values of 0.06 and 0.01 for bacterial and fungal MDS plots, respectively.

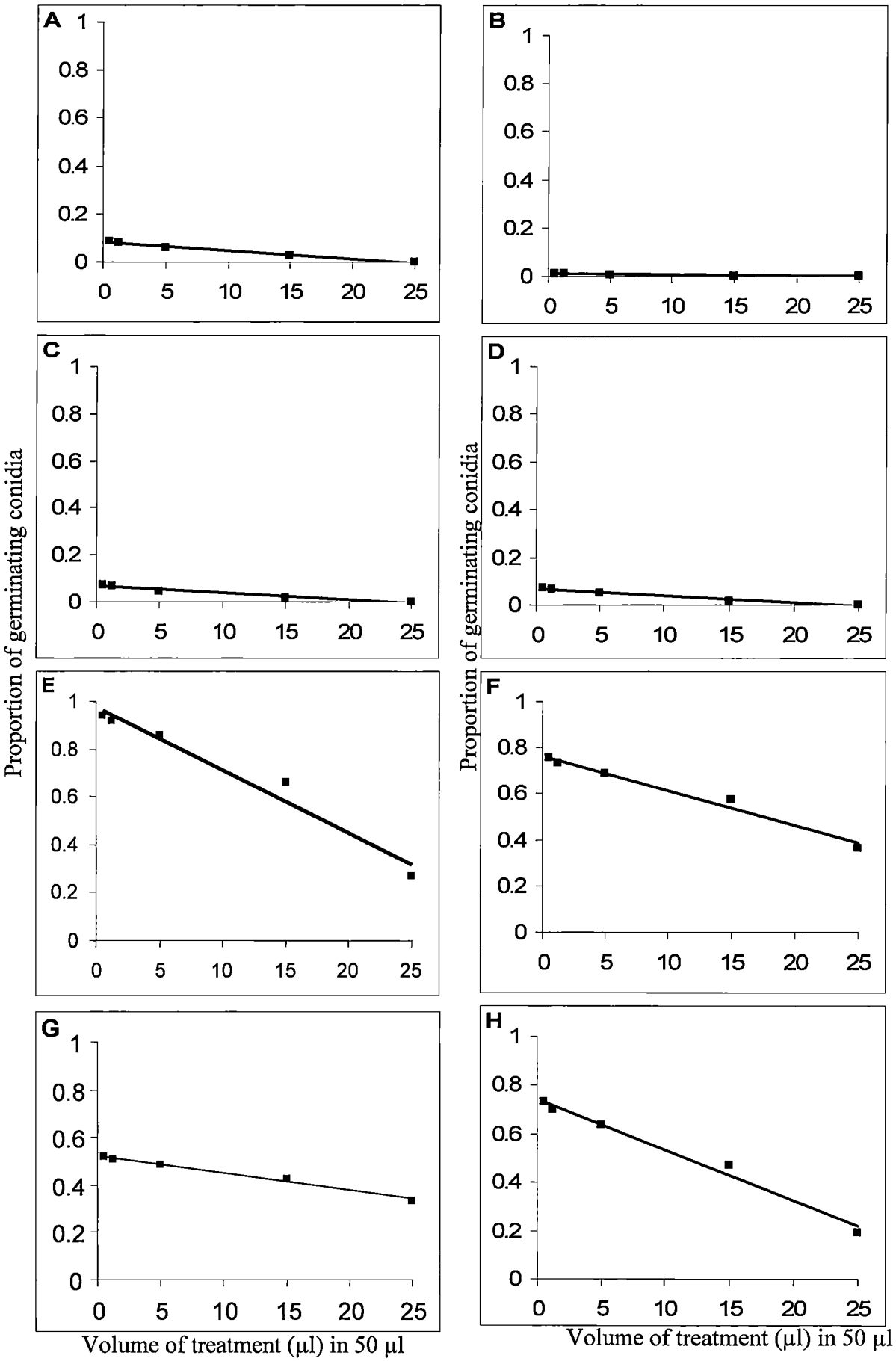
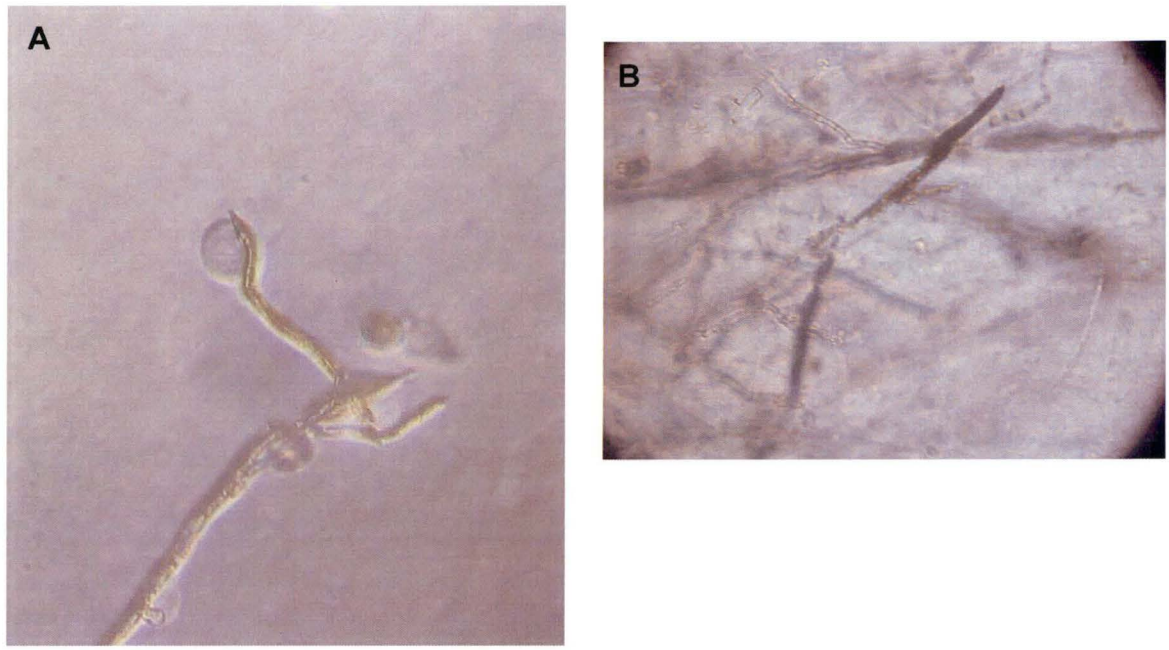


Figure 1.6 Germination proportion of *Botrytis cinerea* conidia *in vitro* when mixed with various dilutions of ACE treatments in sterile water: (A) ACE; (B) ACE + FF; (C) ACE + FF + LK; (D) ACE + LK; (E) Filtered ACE; (F) Filtered ACE + FF; (G) Filtered ACE + FF + LK; and (H) Filtered ACE + LK. FF is Foundation Fish™ added at 24 h at a concentration of 1 part FF:120 parts ACE (v/v). LK is Liquid Kelp™ added at 24 h at a concentration of 1 part LK:60 parts ACE (v/v). ACE was prepared from compost four.

Figure 1.7 Bursting hyphal tip of *Botrytis cinerea* (A) and coagulated hyphal content (B) following contact with filtered ACE in water agar. ACE was prepared from compost four with compost to water ratio of 1:3 and an extraction time of 48 h. Photographs were taken after 7 days incubation in darkness.



1.5 Discussion

The few studies evaluating ACE to date have been based on extracts prepared from mature compost. It was demonstrated, for the first time, that a bacterial dominant ACE prepared from compost prior to maturity consistently inhibited the growth and reproduction of *B. cinerea* on bean leaflets. Specifically, ACEs produced from three different composts sampled in the early secondary mesophilic stage of composting suppressed *B. cinerea* on detached bean leaflets to a greater extent than ACEs produced from compost sampled in later mesophilic stages. Furthermore, an inexpensive and reproducible bean leaflet assay was presented which rapidly and systematically selected the most pathogen-suppressive ACE prior to field tests.

Parameters found to influence the suppression of *B. cinerea* on bean leaflets significantly were the internal windrow temperature and compost age when sampled

for ACE production and the compost to water ratio during extraction. The presence of microorganisms was a substantial component of the activity of ACE and there was limited evidence to suggest that the magnitude of pathogen suppression on bean leaflets was associated positively with the diversity of culturable and non-culturable microorganisms in ACE, as measured by T-RFLP.

Composting is a dynamic process involving complex interactions between chemical, physical and biological factors that led to temporal changes in the relative abundance and diversity of different types of microorganisms (Epstein 1997, Ishii *et al.* 2000, Ryckeboer *et al.* 2003b). In the initial phase of composting, carbon is abundant and microorganisms use carbon as a source of energy for growth and reproduction. As composting proceeds, a large percentage of carbon is converted to carbon dioxide and released by microorganisms. Like carbon, biologically available nitrogen is essential for microbial growth, but if it becomes limiting, then microbial growth may be inhibited, which in turn slows the composting process (Shilesky and Maniotis 1969). Nitrogen was abundant in the compost rows used in this study because salmon waste was added after windrow initiation and thus lowered the C:N ratio. The lack of nitrate in ACE prepared from composts one to three at weeks 5 and 6 indicated that nitrogen was present mostly in organic form, including microbial biomass, and that inorganic nitrogen might have been released from the compost as ammonia and/or nitrate. In general, ACE produced from compost windrows at 7 to 13 weeks old contained nitrate and this observation was in agreement with the findings of other studies where maximum nitrification was noticed in the secondary mesophilic stage of composting (Bishop and Godfrey 1983, Diaz *et al.* 1993). Further research is required to determine if the presence of nitrate in ACE influences its capacity to suppress leaf and fruit pathogens by supporting saprophytic and/or pathogenic

microorganisms on the leaf or fruit surface. Harper *et al.* (1981) found that nitrate or ammonium forms of nitrogen supported abundant growth of *B. cinerea in vitro*. In addition to the C:N ratio, other factors such as aeration rate and moisture can affect composting reactions, highlighting the need for quality control during composting to minimise loss of nitrogen to the external environment and to maximise its conversion to the organic form. It is proposed that a highly pathogen-suppressive ACE might be associated with an absence of nitrate in the extract.

The, pH, dissolved oxygen and soluble salt content of ACE may also influence the degree of disease control in the crop canopy. pH of ACE for each compost age and extraction time was between 6 and 8, which is tolerated by horticultural crops. In most batches of ACE prepared, the amount of dissolved oxygen was above 6 mg/L, which is essential for growth of aerobic microorganisms (Deacon 1997). Anaerobic conditions and numerous acids develop when dissolved oxygen is lower than 6 mg/L (Merrill and McKeon 1998, Bess 2000). The compost used in this study contained high levels of fish waste, which most likely contributed to the relatively high amount of dissolved salts in ACE (> 6 dS/m). If this level of dissolved salts had been present in soil water, then it might have caused a significant decline in the yield of *Vitis vinifera* grapevines (Steppuhn *et al.* 2005). Soil-water (1:1) electrical conductivity (EC) values below 0.8 dS/m, are optimal for crop growth (Anon. 1999). ACE was not applied to soil or potting media in this study and the high salt content did not appear to produce any symptoms related to phytotoxicity on detached bean leaflets. However, more attention should be paid to the soluble salt content of compost and ACE in further experimentation to ensure that sustained application of ACE to the fruit, foliage or soil does not impair plant health, crop yield and quality.

The bean leaflet assay was an efficient and reproducible method for screening production parameters for ACE and it quantified differences among treatments for the growth and reproduction of *B. cinerea*. For example, the magnitude of pathogen suppression by ACE on bean leaflets declined as the amount of compost relative to water used during extraction was reduced (Tables 1.7, 1.8). It was concluded that compost to water ratios of 1:3 to 1:10 should be adopted in field experiments to test the ability of ACE to suppress disease caused by *B. cinerea*. At a ratio of 1:1, the level of pathogen suppression was minimal and probably due to a depleted quantity of dissolved oxygen and hence inactivity of microorganisms (Lasaridi and Stentiford 1998, Al-Dahmani *et al.* 2003).

In compost windrows one and three, maximum pathogen suppression was observed when ACE was prepared from compost with an internal windrow temperature between 40°C and 50°C, whereas the optimum temperature range for compost two was between 30°C and 50°C. The inconsistency in observations between windrow two and the other two rows might be explained by the irregular production conditions for compost two; in particular, the temporal course of the internal compost windrow temperature was atypical because of the discontinuous thermophilic phase (Fig. 1.1). Again, the bean leaflet assay was useful for identifying when to sample the compost windrow for production of a pathogen-suppressive extract. Internal compost windrow temperature is an easy parameter to measure and a practical way of identifying the optimum sampling time. Use of this parameter for producing batches of ACE that consistently suppress *B. cinerea* will depend on using composting conditions that are standardised and monitored rigorously.

The abundance and diversity of microorganisms in ACE, in conjunction with internal compost windrow temperature, was evaluated as another means of selecting compost windrows for production of a pathogen-suppressive ACE. Culture dependent assessment of microbial numbers determines the relative abundance of microorganisms among treatments; however, it does not accurately quantify the absolute change in microbial numbers, given that compost is likely to contain a large proportion of non-culturable microbial species (Tiquia 2005). In general, the numbers of culturable bacteria or fungi did not relate directly to the level of pathogen inhibition on detached bean leaflets. This result was demonstrated clearly in the compost to water ratio experiment, where ACE prepared with a compost to water ratio of 1:1 contained a significantly greater number of fungi and bacteria but significantly reduced pathogen inhibition (Tables 1.7, 1.8). This finding was consistent with the studies of Scheuerell and Mahaffee (2006), who found that the addition of nutrients to ACE increased the number of bacteria but did not reduce grey mould on geranium. There was also no indication in this study of a threshold number of culturable microorganisms in ACE for suppression of *B. cinerea* on bean leaflets, even though a range of cfu (5.2 to 10.4 $\log_{10}$cfu/ml) was observed among extracts assayed. This result suggested that the type and/or diversity of microorganisms rather than their abundance, or the relative abundance of bacteria and fungi in ACE, was important in determining the suppressive ability of compost extract. The exception was ACE produced from compost windrow three, where there was a positive correlation between the PCA1 eigenvector values and the number of culturable fungi (Table 1.7). In this case, both diversity and abundance of fungi may have contributed to a pathogen-suppressive ACE because the highest diversity of fungi was present in ACE prepared when the internal windrow temperature was 48°C (Table 1.7). In aerobic windrow composting, biological diversity, abundance and activity are

primarily determined by windrow temperature (Ryckeboer *et al.* 2003b). The microbial qualities of compost at the early secondary mesophilic stage of composting are described in detail in the General Discussion.

T-RFLP described the dynamics of both culturable and non-culturable microorganisms during the composting process, as well as investigating the diversity of the microbial community in each batch of ACE. Bacterial and fungal diversity, as measured by Total S_{bac} and Total S_{fungi} (Table 1.9), fluctuated as compost cooled. Nevertheless, ACE prepared from compost in the last 3 weeks of composting had significantly less microbial diversity and less impact on *B. cinerea* than ACE prepared from 5-week old compost in the very early secondary mesophilic stage (Table 1.9). These results suggest that T-RFLP can be used to compare the effectiveness of ACE as a function of compost age used to prepare ACE and can indicate the dynamics of the microbial community structure during the composting process. T-RFLP analysis can now be applied to assess the diversity of microorganisms associated with other variables in ACE production, including compost extraction time, compost to water ratio when extraction commences and the impact of varying compost raw ingredients and/or C:N ratios. It is postulated that compost raw ingredients can be varied for production of a pathogen-suppressive ACE, within a defined range of C:N at windrow initiation, as long as windrows are monitored and managed daily to ensure efficient composting and a product that provides consistent results.

ACE directly inhibited the germination of *B. cinerea* conidia *in vitro* and the addition of fish hydrolysate to ACE reduced the mean germination proportion further. A potential mechanism of action by ACE amended with FF is competition for nutrients

because an abundance of nitrogen, amino acids and vitamins in fish hydrolysate (Aspmo *et al.* 2005) would cause an increase in microbial reproduction, activity and numbers, thus creating competition between the microorganisms and the plant pathogen. Competition for nutrients has been proven to be a fundamental mechanism by which biological control agents protect plants from necrotrophic phytopathogens (Blakeman 1993). Fish hydrolysate may have also changed the composition of microorganisms in ACE. Additional *in vitro*, *in vivo* and field experiments are necessary to confirm if there is benefit in amending ACE with fish hydrolysate for pathogen and disease suppression.

In possibly the only other study of the mechanism of action of ACE, Dianez *et al.* (2006) conducted *in vitro* studies of non-filtered, filtered or autoclaved ACE from grape-marc compost for inhibition of nine soil-borne plant pathogens, although maintenance of aerobic conditions was not verified. Dianez *et al.* (2006) found that removal of microorganisms from ACE did not eliminate the suppressive ability of the extract completely, as reported here, suggesting the presence of antimicrobial chemicals in the filtered extract. The lack of negative and positive controls in the study of Dianez *et al.* (2006) for the presence of water soluble antibiotics limits comparison of their results with the findings of this study (Table 1.11, Fig. 1.7). Tetracycline was a very effective antibiotic when used as a positive control (Table 1.11); however, comparing the ability of tetracycline to inhibit the *in vitro* growth of *B. cinerea* to filtered ACE may not allow detection of a low level of antibiotic activity in ACE. If antibiotics against *B. cinerea* in ACE were absent, then one explanation for the activity of filtered ACE was the relatively high salt content. To test this hypothesis, filtered ACE would need to be assayed against a salt solution of similar conductivity and this type of control should be included in future assays.

Nevertheless, the well-cut diffusion assay demonstrated a lack of activity by the ACE filtrates. The combined results for the antibiotic and germination assays, with the latter demonstrating a large difference in activity between unfiltered ACE and filtered ACE treatments, suggests that the filtrate is ineffective in comparison with unfiltered ACE and that the mechanism of inhibition requires close proximity between microorganisms in ACE and *B. cinerea*. The results for the germination assay were consistent with findings from studies where filtered anaerobic compost extracts did not suppress *B. cinerea* on bean leaves (Stindt 1990). Similarly, anaerobic compost extract filtered with paper with decreasing pore sizes increased infection by *Phytophthora infestans* on detached tomato leaves (Ketterer 1990). Additional research is necessary to fully elucidate the mechanism/s of action of ACE. There are likely to be myriad mechanisms involved, with specific mechanisms dominating under certain environmental conditions.

In summary, the growth and reproduction of *B. cinerea* was suppressed on bean leaflets using compost dominated by bacteria and comprising a diverse array of bacterial and fungal taxa. The bean leaflet assay in conjunction with analyses of T-RFLPs were useful tools for evaluating ACE production techniques towards a pathogen-suppressive extract. These tools can now be applied to investigate ACE further in relation to compost prepared from a range of raw materials, as well as the impact of ACE on microbial ecology of leaf and fruit surfaces following application to horticultural crops. Standardised production conditions for ACE, including sampling compost in the early secondary mesophilic stage and a compost to water ratio of 1:3, have been adopted in field experiments to evaluate ACE for the management of powdery mildew and botrytis bunch rot in wine grapes (Chapter 2).

CHAPTER 2:

STANDARDISED AEROBIC COMPOST EXTRACT (ACE) SUPPRESSES POWDERY MILDEW AND BOTRYTIS BUNCH ROT UNDER GLASSHOUSE AND VINEYARD CONDITIONS

2.1 Abstract

Sustainable alternatives to synthetic fungicides are sought because of the potential for pathogens to evolve fungicide resistance, negative impacts on beneficial organisms and concerns about human exposure to fungicide residues. Aerobic compost extract (ACE), a watery extract of compost, was evaluated for the management of grapevine powdery mildew and bunch rot caused by pathogens of different biology and epidemiology; namely, the obligate biotroph *Erysiphe necator* and the nectrotroph *Botrytis cinerea*. ACE or ACE amended with fish hydrolysate and/or liquid kelp was prepared using standardised methods (1:3 compost:water ratio, Chapter 1) and applied nine or 12 times at 10-14 day intervals to Chardonnay or Riesling vines grown commercially in different growing seasons in southern Tasmania. Powdery mildew was controlled by ACE or amended ACE to a commercially acceptable level on Chardonnay leaves and bunches under conditions of high disease severity. The incidence of latent *B. cinerea* in Chardonnay bunches at harvest, after moist incubation, was nearly half that observed in non-treated bunches. The incidence and severity of sporulation of *B. cinerea* on Riesling bunches was reduced significantly by ACE or amended ACE relative to a dechlorinated water control treatment; these bunches escaped visible infection by *E. necator* but powdery mildew was controlled on leaves. Treatment of Riesling leaves with ACE increased the number of culturable microorganisms on leaves 100-fold, 1 h after application. By 13 days post-application the number of culturable microorganisms remained

higher than pre-application counts. Under glasshouse conditions, the mean powdery mildew severity on Cabernet Sauvignon leaves was less than 0.1% when ACE was applied up to 4 days before or up to 7 days after inoculation with *E. necator* conidia; mean severity on non-treated, inoculated leaves was 22%. This result suggested that ACE had curative as well as protective properties. Further research is required to determine mechanisms of action, effectiveness in a range of environments and spray timing in relation to pathogen activity and host susceptibility to disease.

2.2 Introduction

Botrytis bunch rot, caused by *B. cinerea*, and powdery mildew, caused by *E. necator,* are two fungal diseases renowned in the wine grape industry for inciting considerable losses in grape yield and wine quality (Stummer *et al.* 2005, La Guerche *et al.* 2006). These diseases are commonly managed by regular applications of protective fungicides. However, the number of pathogen populations reported to tolerate various synthetic fungicides is increasing (Brent and Holloman 2007) and there has been a global push in recent decades towards sustainable disease management and alternative measures for crop protection (for example; Yildirim *et al.* 2002, Crisp *et al.* 2006a).

Aerobic compost extract (ACE) is a watery extract of compost that is receiving increased attention in horticulture as a substitute for commercial fungicides or as a component of integrated disease management. Anecdotal reports describe a reduction in the severity of numerous diseases when ACE is applied as a foliar spray to various crops (Scheuerell and Mahaffee 2002, Litterick *et al.* 2004). In contrast, scientific evaluation of ACE for managing a diverse range of fungal diseases and crops demonstrated no significant reduction in disease severity when compared to non-

treated control treatments (Welke 2004, Al-Mughrabi 2006, Scheuerell and Mahaffee 2004, 2006, Sturtz *et al.* 2006). According to Haggag and Saber (2007), ACE prepared to their specifications could suppress early blight on tomato (*Alternaria solani*) and onion (*A. porri*) to the same degree as conventional treatments. However, the method of compost extraction was not described and it is not known whether or not aerobic conditions were maintained during production.

Methods of ACE production either on-farm or for commercial sales have been variable and manipulated in an effort to increase disease suppression (Weltzien 1992, Scheuerell and Mahaffee 2002). Standardisation of the production variables is essential for maximum and consistent disease control. Compost source, time since compost windrow initiation, internal windrow temperature, compost extraction time and various extract physical and chemical characters were evaluated and particular variables standardised (Chapter 1) to produce an ACE that consistently suppressed the growth and sporulation of *B. cinerea* on detached bean leaflets. Despite variation in raw ingredients used to prepare compost, a pathogen-suppressive ACE was reproduced from compost with an internal temperature of between 40°C and 50°C in the cooling phase of composting. The level of *B. cinerea* suppression in one compost windrow sampled over time during the cooling phase appeared to be correlated to the diversity of bacteria and fungi according to analysis of terminal restriction fragment length polymorphisms.

The main aim of the research described in this Chapter was to evaluate the effectiveness of multiple applications of standardised ACE (Chapter 1) for managing powdery mildew and botrytis bunch rot in commercial vineyards in southern Tasmania, Australia. Within this general aim, two specific objectives were

addressed toward improved understanding of the mechanism of action of ACE. The first objective was to investigate changes in the abundance of culturable microorganisms in the phyllosphere of grapevine leaves following application of ACE under field conditions. The second objective was to quantify the level of powdery mildew suppression by ACE in relation to the time of application before or after inoculation of leaves of potted grapevines with *E. necator* conidia. The results demonstrate the significant potential for ACE to be incorporated into a management program for grapevine powdery mildew and botrytis bunch rot.

2.3 Materials and Methods

2.3.1 Production of ACE

Compost extract was produced according to the pilot-scale technique described in Chapter 1. In short, compost was prepared with an initial C:N ratio of 30:1, with raw ingredients varying but based on a combination of cow manure or chicken manure and/or timber waste as described in Chapter 1. Waste from the commercial culture of Atlantic salmon (*Salmo salar*) in Tasmania was added to each compost windrow 2 weeks after windrow initiation. For production of extract, compost was sampled when the internal windrow temperature was 50°C in the cooling phase of composting and extracted in a 1:3 ratio with dechlorinated (Section 1.3.2), aerated water for 48 h to produce aerobic compost extract (ACE). Previously (Chapter 1), compost:water ratios of 1:3 and 1:10 suppressed *B. cinerea* on bean leaflets to an equivalent level. A compost:water ratio of 1:3 was selected for this study because this ratio was being used in commercial production of ACE. Two variations on ACE were also prepared whereby one or two additional nutrients were added 24 h after extraction was initiated. The nutrients were hydrolysate of Atlantic salmon (Table 1.2) and liquid bull kelp (*Durvillaea potatorum*), formulated by Soil First Pty Ltd, Margate,

Tasmania, as Foundation Fish™ (FF) and Liquid Kelp™ (LK). Nutrient concentrations were 1 part FF: 120 parts ACE (v/v) and 1 part LK: 60 parts (v/v). The FF additive was not included in amended ACE in 2006/07, due to the possibility of the re-growth of human pathogens (refer to Chapter 3). At 48 h extraction time, immediately prior to field application, each batch of ACE or modified ACE was analysed for pH, dissolved oxygen, conductivity, temperature and nitrate. Nitrate was determined from nitrate Merckoquant® strip tests (Merck Pty Ltd). The other physical parameters were measured using WTW Handheld 340i or Inolab meters (Merck Pty Ltd). For the glasshouse experiment, all physical parameters were measured following production and before storage as specified in the next section. One batch of ACE per field experiment was also sent to the Environmental Analysis Laboratory (NSW, Australia) for analysis of macro and micronutrients.

2.3.2 Glasshouse experiment

A glasshouse experiment was conducted to identify the level of powdery mildew suppression by ACE in relation to the period of time before or after infection of leaves by *E. necator*. *Vitis vinifera* cv. Cabernet Sauvignon, clone LC14, was propagated from hardwood cuttings obtained from South Australian Vine Improvement, Nuriootpa, South Australia. Roots were initiated by application of 2 g/L iso-butyric acid to the cutting base before planting into 15 cm-deep, moist vermiculite with basal nodes 3 cm above a heat bed (Thermofilm, Victoria) maintained at 25°C. The heat bed with cuttings was placed in a cool storeroom (< 8°C) to retain dormancy of the buds during root initiation. After 22 days, individual cuttings with roots were planted into 20 cm-diameter pots containing a potting mixture prepared specifically for grapevines: three parts pine bark, one part coarse sand, one part fine sand, 2 g/L Osmocote® Classic controlled release fertiliser for

native plants, 1.5 g/L calcium carbonate and 1.5 g/L magnesium sulphate (Horticultural Supplies, Tasmania, Australia). Potted vines were maintained on raised mesh tables at 25°C ± 4°C in a glasshouse and watered regularly. Infection by *E. necator*, prior to inoculation, was prevented by release of vapours of penconazole (TOPAS® 100EC, Syngenta Australasia, Szkolnik, 1983).

When vines had developed at least six leaves, the six youngest, fully expanded leaves were designated a position; namely, leaf position one for the youngest expanded leaf, leaf position two for the next oldest leaf, and so on. At this time and immediately prior to the first treatment of vines with ACE, experimental vines were transferred to a glasshouse maintained at 19°C ± 2°C where vapours of penconazole were absent. The vines were placed 30 cm apart on an elevated table and a shade cloth with 70% UV protection was positioned 1 m above the table and draped over the sides of a support surrounding the potted vines to increase humidity and reduce light intensity.

ACE was prepared, as described previously, from compost based on timber and fish waste with a compost to water ratio of 1:3. Six 500 ml samples of ACE were stored at 4°C for up to 14 days prior to use. The experiment was designed as a randomised complete block design with six blocks and eight treatments. Each plot comprised a single potted grapevine. The treatments were: ACE applied 7, 4 and 1 day/s prior to inoculation with *E. necator* and ACE applied 1, 4 and 7 day/s post-inoculation. Control treatments were a dechlorinated water control applied 1 day post-inoculation and a non-treated control. ACE was removed from cool storage and upon reaching room temperature (21°C ± 4°C) was applied immediately as a fine mist to the adaxial side of the leaves using a hand-held atomiser (Hills™ Trigger).

A bulk isolate of *E. necator* was collected from Vineyard B (see Section 2.3.3.2) in February 2004 and multiplied using detached leaves of *Vitis vinifera* cv. Cabernet Sauvignon, clone LC10, as described by Evans *et al.* (1996). Conidia were harvested from 14 day-old powdery mildew colonies by shaking two infected leaves in 50 ml of distilled water containing 0.05 % (v/v) Tween 20. The concentration of conidia was adjusted to approximately 1 x 10^5 conidia per ml of sterile distilled water with the aid of a haemocytometer. Conidial suspensions were applied with a handheld atomiser (Preval® sprayer power unit, Precision Valve Corporation) until droplets of water were visible on the leaves. Following inoculation of all vines, a fan was placed near the vines until all water droplets had dried.

Powdery mildew severity and incidence on the leaves tagged at the beginning of the experiment was recorded at 25 days post-inoculation, to account for the fact that the glasshouse temperature was below the optimum of 24-25°C for development of powdery mildew (Delp 1954). Incidence was calculated as the percentage of leaves with powdery mildew at a particular leaf position across the six blocks (n = 6 per leaf position). Severity was assessed for individual leaves at each leaf position using a standard area diagram (B. Emmett, Department of Primary Industries, Victoria, personal communication). Mean severity was then calculated for the three youngest tagged leaves per plant because these leaves represented the zone of the shoot that expressed greatest disease severity, given that *Vitis vinifera* expresses leaf-age related resistance to powdery mildew (Doster and Schnathorst 1985). The incidence of powdery mildew for the three youngest leaves was calculated from the data for severity.

2.3.3 Field experiments

Two field experiments to assess the effectiveness of ACE in managing powdery mildew, caused by *E. necator*, and bunch rot caused by *B. cinerea*, were conducted in different growing seasons at two different vineyards in the Coal River Valley of southern Tasmania, Australia. One vineyard was managed organically which enabled direct comparison of the application of ACE to a standard organic spray program for powdery mildew. This organic vineyard rarely developed severe botrytis bunch rot. Therefore, a conventionally-managed vineyard was selected with a history of moderate to severe botrytis bunch rot to evaluate application of ACE for suppression of this disease in relation to a conventional spray program for both powdery mildew and botrytis bunch rot. This change also necessitated selection of a different cultivar for evaluation of ACE.

2.3.3.1 *Chardonnay in 2005/06*

In 2005/06, the field experiment was at Vineyard A (42°44' S, 147°29' E) located in Penna, Tasmania, in a block of *V. vinifera* cv. Chardonnay managed organically. The trellising system was Vertical Shoot Positioning (VSP), with rows 2.5 m apart and vines 1.5 m apart. Rows were oriented north east to south west. The trial was a randomised complete block design with four treatments and five replicates of plots with six to seven vines. The treatments were ACE, ACE amended with FF and LK, a standard organic fungicide program (Table 2.1) and a non-treated control.

There were two buffer rows of vines separating the trial area from the rest of the vineyard block to capture spray drift; these were hand sprayed with a standard organic spray program. Spraying was completed in the morning on days of no rain and all treatments were applied with a hand held gun attached to a hose reel and

diaphragm pump. The pump pressure was 1,500-1,600 kPa, delivering approximately 63 ml per s. The vines were sprayed nine times, every 10-14 days from Eichorn-Lorenz (E-L, Coombe 1995) stage 13 to E-L stage 34 (Table 2.1). Ambient temperature, rain and relative humidity were recorded using a Measurement Engineering Australia (MEA) Automated Weather Station (AWS) positioned 100 m north of the trial site. Weather data are presented in Fig. 2.1A.

Once colonies of *E. necator* were detected, 20 leaves or ten bunches were selected randomly, from three central vines per plot, for fortnightly assessment of the mean disease severity per plot. The severity of powdery mildew on a bunch or leaf was assessed using a standard area diagram (B. Emmett, Department of Primary Industries, Victoria, personal communication). Disease incidence was the percentage of leaves or bunches with powdery mildew per plot and was derived from the measures of disease severity. The final disease assessment occurred at veraison, when maximum differences among experimental treatments were likely to be evident.

The severity and incidence of bunch rot caused by sporulating *B. cinerea* was assessed at harvest for 30 bunches selected randomly per plot, with the aid of a standard area diagram (B. Emmett, Department of Primary Industries, Victoria, personal communication). The level of latent botrytis infection was also assessed at harvest. Ten bunches on each side of the panel were harvested per plot and transferred by road in a cooler box for 30 min to New Town Research Laboratories. The 20 bunches were surface sterilised in 70% ethanol for 30 s, then 2 min in 0.35% sodium hypochlorite and 30 s in 70% ethanol and air dried (Coertze *et al.* 2001). Each bunch was placed separately into plastic containers on sterile paper towel

soaked in distilled water to maintain high humidity. The containers were maintained at room temperature (21°C ± 4°C) on the laboratory bench. After 10 days, bunches were scored for incidence and severity of sporulating colonies of *B. cinerea.*

Table 2.1. Materials applied to Chardonnay vines in the standard organic fungicide program at vineyard A in 2005/06. ACE or amended ACE treatments were applied on the same dates.

Date	Crop stage[1]	Spray volume per vine	Material, manufacturer and rate of active constituent[2] per 100 L
17-Oct	E-L 13	0.29 L	Acadian™, Bio-Ag Consultants and Distributors Inc., 160 g (kelp meal)
28-Oct	E-L 15	0.29 L	Acadian™ plus Synetrol Horti® oil, Organic Crop Protectants Australia, 200 ml (Australian canola oils and essential botanical oils)
9-Nov	E-L 16	0.34 L	Acadian™ plus Synetrol Horti® oil
21-Nov	E-L 19	0.29 L	Cosavet™ DF, Sulfur Mills Ltd, 800 g (colloidal sulfur)
1-Dec	E-L 23	0.51 L	Cosavet™ DF
9-Dec	E-L 26	0.86 L	Cosavet™ DF
19-Dec	E-L 28	0.57 L	Acadian™ plus Synetrol Horti® oil
29-Dec	E-L 30	0.57 L	Synetrol Horti® oil
15-Jan	E-L 31	0.57 L	Synetrol Horti® oil

[1] Crop stage determined according to a modified Eichorn-Lorenz scale (Coombe 1995).
[2] Rate of active constituent for each material remained the same throughout trial period.

The mean total soluble solids (°Brix) per treatment was measured at harvest. Five berries from each of five basal bunches were sampled from two vines per plot, adjacent to the vines used for disease assessment. Juice was extracted from each sample of five berries and a droplet of juice placed on the well of a refractometer (PAL - 1 Digital Refractometer, ATAGO™) to measure °Brix for the calculation of mean °Brix per plot.

2.3.3.2 *Riesling in 2006/07*

In 2006/07, the field experiment was at Vineyard B (42°36' S, 147°26' E) located about 6 km north of Campania, Tasmania, approximately 30 km north-west and inland from the slightly cooler 2005/06 site. The trial site was managed conventionally and located at the south-eastern and elevated end of a block of *V. vinifera* variety Riesling because a severe frost in October 2006 damaged vines at lower elevation in the block. Vines were 1 m apart and trellised by VSP with rows 2.5 m apart, oriented north-north/west to south-south/east. Dataloggers (Tinytags, Hastings Data Loggers, NSW, Australia) that recorded temperature, relative humidity and rainfall were positioned at a height of 1.5 m in open grassland approximately 50 m from the trial site. Weather data are summarised in Fig. 2.1B.

The trial was a randomized complete block design with six blocks of 12 treatments for evaluating various crop protection programs for botrytis bunch rot. Each block comprised a single vineyard row and each plot contained seven to eight vines. There were two rows of buffer vines on either side of the trial site and a buffer zone of seven to eight vines at the northern boundary of the site where rows extended into the non-trial area of the block. Only five of the 12 treatments were used for the evaluation of ACE, including unamended ACE, ACE amended with LK, a control treatment of dechlorinated water and two standard fungicide programs; one for powdery mildew only and one for both powdery mildew and botrytis bunch rot (Table 2.2). Treatments were applied by hand as described previously, except the pump pressures were 1,500–1,600 kPa from E-L 12 to E-L 19 and 1,000 kPa from E-L 19 to E-L 37. ACE treatments were applied 12 times throughout the season, every 10 to 14 days from E-L 12 until E-L 34.

The incidence and severity of powdery mildew was assessed as described previously. Unlike the previous season, latent infection by *B. cinerea* was assessed at pre-bunch closure. Five basal bunches and five distal bunches were sampled randomly per plot and transported by road in cooler boxes for 40 min to the laboratory. A basal bunch was the lowest bunch on a shoot trained upwards and a distal bunch was the bunch above the basal bunch. Bunches were frozen overnight and then each bunch was surface sterilised as described previously. Twenty berries with pedicels attached were excised from each bunch and placed in separate cells of Nylex^{TM} Gutter Guard in plastic containers lined with paper towel soaked in sterile distilled water to maintain high humidity. The lids were replaced and the containers maintained at room temperature (21°C ± 4°C) on a laboratory bench, with natural light from windows, for 14 days. Each berry was then examined for *B. cinerea* sporulation at 40 x magnification and the incidence of latent infection expressed as the percentage of the 20 berries with sporulating *B. cinerea* (Beresford and Hill 2008).

The severity of visible bunch rot and sporulating *B. cinerea* per bunch was assessed with the aid of a standard area diagram (B. Emmett, Department of Primary Industries, Victoria, personal communication) four times between veraison and harvest. For the first assessment 30 bunches were selected randomly from the central five or six vines per plot and then the same 30 bunches were assessed for subsequent assessments. At harvest, all bunches from the central vine per plot were collected and weighed. Yield per plot, expressed as the equivalent tonnes of grapes per hectare (ha), was calculated as the number of vines per ha multiplied by the number of bunches per central vine multiplied by the average bunch weight. Ten of the 30 tagged bunches were collected from each plot and bunch compactness was calculated according to the water displacement method (Shavrukov *et al.* 2004). Juice was

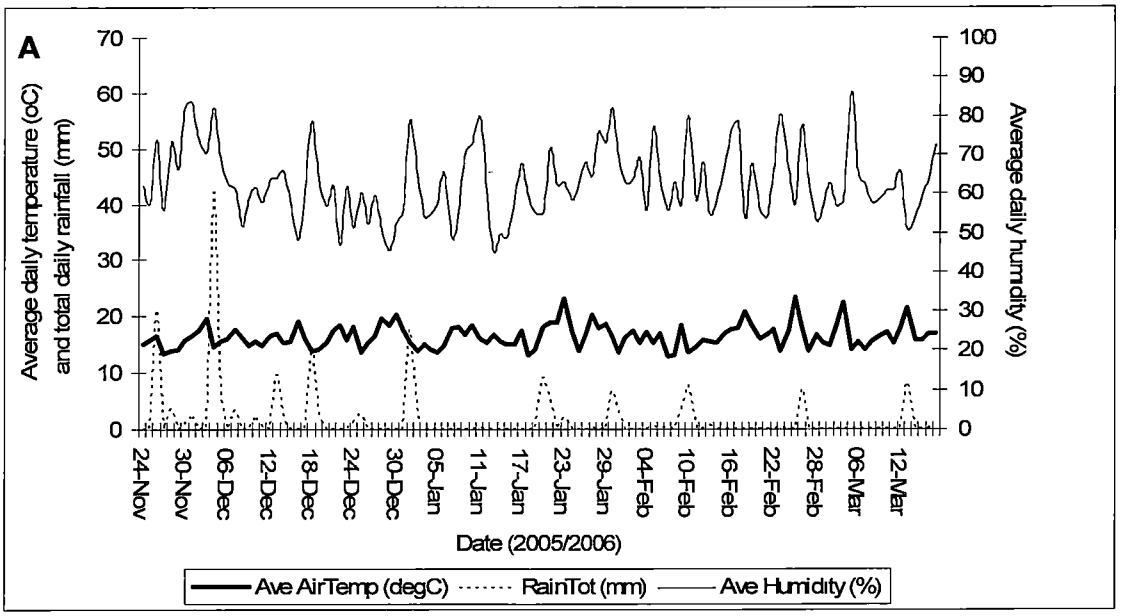
extracted from the same ten bunches and used to measure total soluble solids (^{o}Brix), Titratable Acidity (TA) and pH according to the procedures described by Iland *et al.* (1996).

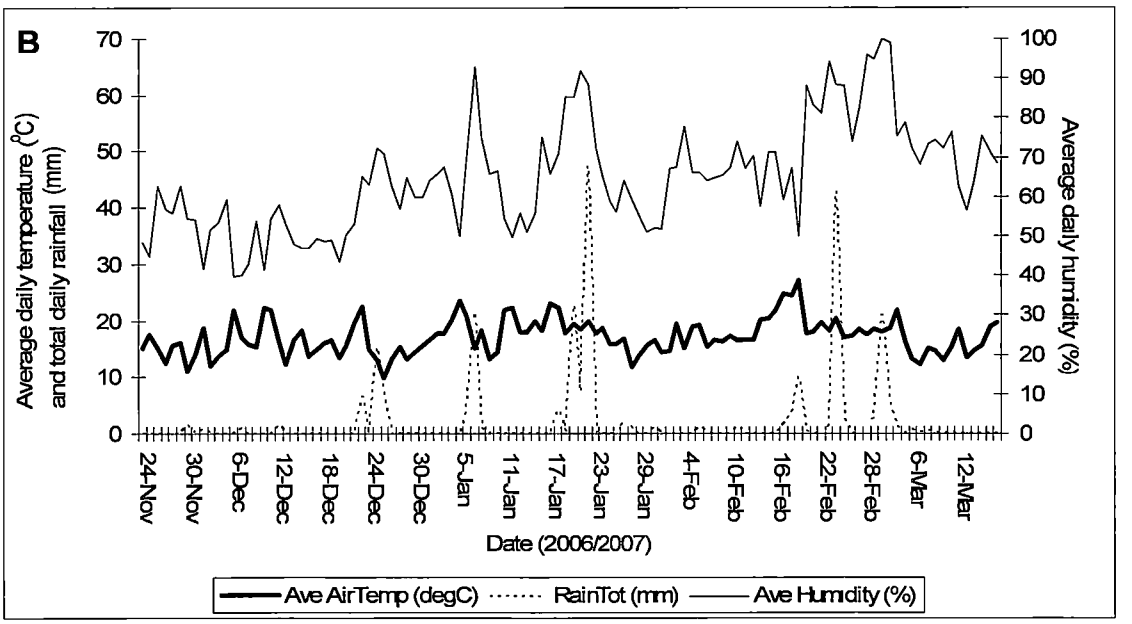
Table 2.2 Materials applied to Riesling vines at Vineyard B, 2006/07, in the standard fungicide program for powdery mildew and botrytis bunch rot. ACE or amended ACE treatments were applied on each date except that the final treatments of ACE were applied on February 21, 2007.

Date	Crop stage[1]	Spray volume per vine	Powdery or downy mildew fungicide and rate of active constituent[2] per 100 L	Fungicide for botrytis bunch rot and rate of active constituent[2] per 100 L
19-Oct	E-L 12	0.31 L	Prill Dry Champ® WG, Nufarm Americas Inc., 180g (cupric hydroxide)	
1-Nov	E-L 14	0.31 L		
13-Nov	E-L 15	0.63 L		
16-Nov	E-L 16	0.31 L	Cosavet™ DF, Sulfur Mills Ltd, 600g (colloidal sulfur), Prill Dry Champ® WG	
23-Nov	E-L 18	0.54 L	Topas® 100 EC, Syngenta Crop Protection Pty Ltd, 12.5ml (penconazole)	Bravo® 500, Syngenta Crop Protection Pty Ltd., 200ml (chlorothalonil)
27-Nov	E-L 22	0.52 L		Scala® 400 SC, Bayer CropScience, 200ml (pyrimethanil)
4-Dec	E-L 24	0.50 L		
4-Jan	E-L 26	0.52 L	Flint® 50 WG, Syngenta Crop Protection Pty Ltd, 14g (trifloxistrobin)	
16-Jan	E-L 31	0.60 L	Cosavet™ DF	Captan 900 WG, Crop Care Australasian Pty Ltd., 120g (captan)
31-Jan	E-L 32	0.54 L		
21-Feb	E-L 34	0.42 L	Cosavet™ DF	Switch 62.5 WG, Syngenta Crop Protection Pty Ltd, 80g (cyprodinil and fludioxonil), Sync™ Fungicide Activator, Precision Laboratories Inc., 20ml (carbohydrate based surfactant, an amine polymer complex and a pH buffer)
15-Mar	E-L 37	0.40 L	Cosavet™ DF	Rovral® Liquid Fungicide, Bayer CropScience, 100ml (iprodione)

[1] Crop stage determined according to a modified Eichorn-Lorenz scale (Coombe 1995).
[2] Rate of active constituent for each material remained the same throughout trial period.

Figure 2.1 Average daily temperature (°C), relative humidity (%) and total daily rainfall (mm) at (A) Vineyard A, 2005/06 at (B) Vineyard B, 2006/07.





2.3.3.3 *Microbial abundance in the phyllosphere*

At both vineyard sites, microbial populations were sampled from leaves in plots treated with ACE at E-L stage 34 (veraison). At Vineyard A, six leaves were sampled randomly from each plot, 30 min before application of ACE, and 1 h, 1 day and 5 days after application of ACE, and 1 day before the subsequent spray. The sampling strategy was the same at Vineyard B, except that leaves from the ACE treatment and three other treatments specified in Table 2.13 were also collected one day before subsequent spray application. The leaves were placed in plastic bags and transported to the laboratory in a cooler box. Ten segments were taken from each leaf, five on either side of the midrib, with an 11 mm sterile cork borer (Met-App Pty Ltd). These sections were placed in 10 ml of sterile saline solution (0.32 M sodium chloride) and vortex mixed for 1 min. A serial dilution was performed in saline solution and 100 µl of dilutions were spread onto yeast peptone dextrose agar (YPDA, selective for yeasts), acidified potato dextrose agar (APDA, selective for filamentous fungi) or nutrient agar (NA to enumerate bacteria). The NA plates were inverted and incubated at 25°C for 72 h. The YPDA and APDA plates were placed in the dark at 21°C ± 4°C) for 7 days. The number of colony forming units (cfu) of bacteria, yeast and fungi was calculated per ml of solution.

2.3.4 Data analyses

Data were subjected to analysis of variance (ANOVA) in Genstat□ for Windows 8th Edition. When necessary, percentages were arcsine transformed and colony forming units (cfu) were transformed to $\log_{10}$cfu/ml. In all tables of results, df is the degrees of freedom and lsd is the least significant difference.

2.4 Results

2.4.1 Physical and chemical characters of ACE

The mean physical and chemical characters of extracts applied to glasshouse vines were unlike those applied in the vineyards (Table 2.3). In the glasshouse, the concentration of dissolved oxygen appeared to be higher and conductivity was lower than the field. In the field trials, the mean pH of ACE or amended ACE was close to neutral, the mean temperature was 23–26°C, the mean dissolved oxygen was always greater than 6.1 mg/ L and the mean conductivity was 5.0–6.7 dS/m. Nitrate (0–100 mg/L) was present in all extracts prepared for application at Vineyard B. When compared to unamended ACE, there appeared to be a small but insignificant increase in conductivity when ACE was amended with either LK or FF and LK (P = 0.46 and 0.65, respectively).

Table 2.3 Average selected physical and chemical characters of 48-h ACE and amended ACE measured before storage and application in the glasshouse or field, respectively. Each value for Vineyard A in 2005/06 and Vineyard B in 2006/07 was the average for nine and 12 batches of ACE, respectively, corresponding to the different times of application

Physical and chemical properties	Glasshouse experiment	Vineyard A		Vineyard B	
	ACE	ACE	ACE with FF[1] and LK[2]	ACE	ACE with LK
pH	7.1	7.5	7.4	7.2	7.2
oxygen (mg/L)	8.2	6.8	6.1	6.9	7.1
conductivity (dS/m)	1.0	6.3	6.7	5.0	5.6
temperature (°C)	23	27	27	24	24
nitrate (mg/L)	0	0	0	50	50

[1] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v).
[2] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

The total quantities of various macronutrients applied to vines treated with ACE during 2005/06 and 2006/07 are shown in Table 2.4. The total quantity of nitrogen applied in 2005/06 at Vineyard A (14.8 kg/ha/year) was much greater than the total quantity (4.7 kg/ha/year) applied in 2006/07 at Vineyard B.

Table 2.4 Total nutrients applied to vines from multiple applications of ACE at Vineyard A in 2005/06 and at Vineyard B in 2006/07.

Nutrient	Total quantity of nutrient applied (kg/ha/year)	
	Vineyard A	Vineyard B
nitrogen	14.8	4.70
phosphorus	2.26	0.64
potassium	16.2	15.8
calcium	0.49	1.45
magnesium	0.53	1.09
chloride	28.3	25.2
sulfate	6.36	2.75

2.4.2 Glasshouse experiment

Inoculation of leaves of glasshouse grown Cabernet Sauvignon vines with *E. necator* confirmed the expression of leaf age-related resistance to powdery mildew. Leaves in positions one and six represented the youngest and oldest leaf, respectively. In shoots that were inoculated but were not treated with ACE, the incidence and severity of powdery mildew on leaves declined with increasing leaf position (Table 2.5). The greatest mean incidence and severity of powdery mildew was evident on non-treated and dechlorinated water treated leaves (Table 2.6). There was a significantly greater incidence and severity of powdery mildew on leaves treated with ACE 7 days prior to *E. necator* inoculation than with the other times of ACE application.

Table 2.5 The percentage incidence and mean severity of powdery mildew on 6 non-treated leaves per leaf position of *Vitis vinifera* variety Cabernet Sauvignon that had been inoculated with *Erisiphe necator* conidia in the glasshouse 25 days previously. Leaves in positions one and six represent the youngest and oldest leaf, respectively.

Leaf position	Incidence (%)	Mean Severity (%)
1	100	21
2	100	36
3	100	8.2
4	40	1.4
5	40	0.4
6	0	0.0

Table 2.6 Effect of different treatment times with ACE on mean incidence and severity of powdery mildew on leaves of *Vitis vinifera* variety Cabernet Sauvignon in the glasshouse, 25 days after inoculation with *Erisiphe necator* conidia. Arcsine-transformed data in parentheses. Means within columns followed by the same letter are not significantly different at $P = 0.05$.

Spray timing in relation to inoculation with *E. necator* conidia	Mean incidence (%)		Mean severity (%)	
-7 days	46.7 (0.75)	b	2.75 (0.05)	b
-4 days	6.67 (0.12)	a	0.07 (0.00)	a
-1 days	0.00 (0.00)	a	0.00 (0.00)	a
+1 days	6.67 (0.12)	a	0.01 (0.00)	a
+4 days	0.00 (0.00)	a	0.00 (0.00)	a
+7 days	13.3 (0.25)	a	0.02 (0.00)	a
Inoculated but not treated	100 (1.57)	c	21.7 (0.24)	c
Dechlorinated water applied at + 1 day	100 (1.57)	c	21.5 (0.24)	c
lsd for transformed means	0.44		0.05	
df	28		28	
P	< 0.001		< 0.001	

2.4.3 Field experiments

2.4.3.1 *Suppression of powdery mildew on leaves*

At Vineyard A, powdery mildew was observed on the abaxial surface of leaves in non-treated plots prior to capfall when the inflorescence was well developed(Fig.

2.2). Incidence on the abaxial surface of leaves increased to 100% by E-L stage 29 (berries pepper-corn size) and the infection progressed to the upper surface of leaves by E-L stage 23 (17-20 leaves separated, 50% caps off). Powdery mildew infection on Riesling leaves at Vineyard B was considerably later in 2006/07, when compared with Chardonnay in 2005/06 (Fig. 2.2). Powdery mildew was first observed on the abaxial surface of Riesling leaves in the dechlorinated water control plots when the berries were pea-sized, reaching a maximum of 55% incidence by harvest. Powdery mildew progressed to the adaxial surface of leaves after veraison (Fig. 2.2).

All treatments in both growing seasons significantly reduced the incidence and severity of powdery mildew on the adaxial surface of leaves when compared to the non-treated or dechlorinated water controls (Table 2.7). When the dechlorinated water control was excluded from analysis of treatments at Vineyard B in 2006/07, the incidence of powdery mildew on the adaxial surface of leaves treated with ACE plus LK was significantly lower ($P = 0.02$) than observed on vines treated with ACE or the standard program.

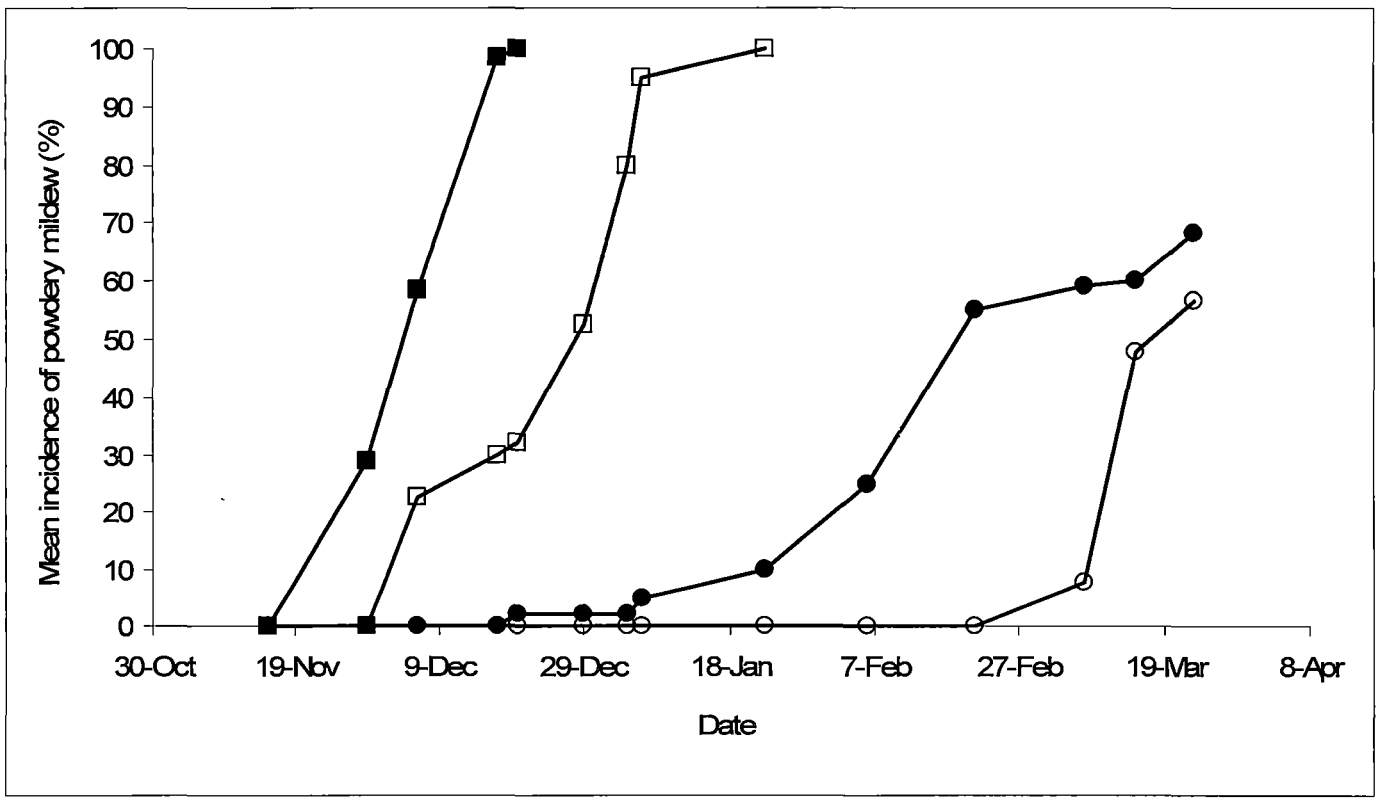


Figure 2.2 Mean incidence of powdery mildew on (a) the abaxial (■) and adaxial (□) surface of Chardonnay leaves at Vineyard A from November 15, 2005, when the inflorescence was well developed prior to cap fall (E-L 17) and (b) the abaxial (●) and adaxial (○) surface of Riesling leaves at Vineyard B between December 20, 2005 (E-L 31, berries pea size) and March, 23, 2006 (E-L 37, berries not quite ripe).

Table 2.7 Mean incidence and severity of powdery mildew on the adaxial surface of leaves at Vineyard A, E-L 31 (pea size berries) and Vineyard B, E-L 37 (berries not quite ripe). Transformed data in parentheses. Means within columns for transformed data followed by the same letter are not significantly different at $P = 0.05$.

Treatment	Mean incidence (%)				Mean severity (%)			
	Vineyard A		Vineyard B		Vineyard A		Vineyard B	
Non-treated (Vineyard A) or dechlorinated water control (Vineyard B)	100 (1.57)	b	56.7 (0.92)	b	73.1 (1.09)	b	23.7 (0.19)	b
ACE	4.5 (0.18)	a	7.9 (0.21)	a	0.1 (0.07)	a	0.5 (0.01)	a
ACE with FF[1] and LK[2] (Vineyard A) or ACE with LK (Vineyard B)	6.0 (0.18)	a	1.3 (0.05)	a	0.1 (0.05)	a	0.0 (0.00)	a
Standard fungicide program for powdery mildew	5.5 (0.23)	a	11.3 (0.34)	a	0.1 (0.02)	a	0.0 (0.00)	a
lsd for transformed means	0.14		0.37		0.10		0.12	
df	12		15		12		15	
P	< 0.001		< 0.001		< 0.001		= 0.012	

[1] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v).
[2] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

2.4.3.2 *Suppression of powdery mildew on fruit*

Powdery mildew on non-treated Chardonnay bunches in 2005/06 was severe, whereas Riesling bunches escaped infection by *E. necator* in 2006/07. The incidence of powdery mildew on non-treated Chardonnay bunches increased from close to 0% at E-L 26 (cap-fall complete) to 100 % by E-L 31 (berries pea size) (Fig. 2.3). An increase in powdery mildew severity followed the increase in incidence and the maximum severity observed was 77% (Table 2.8). Both ACE treatments significantly reduced the incidence and severity of powdery mildew on Chardonnay bunches when compared to the non-treated control and were equivalent to the standard organic spray program, which reduced powdery mildew severity to a commercially acceptable level (Table 2.8).

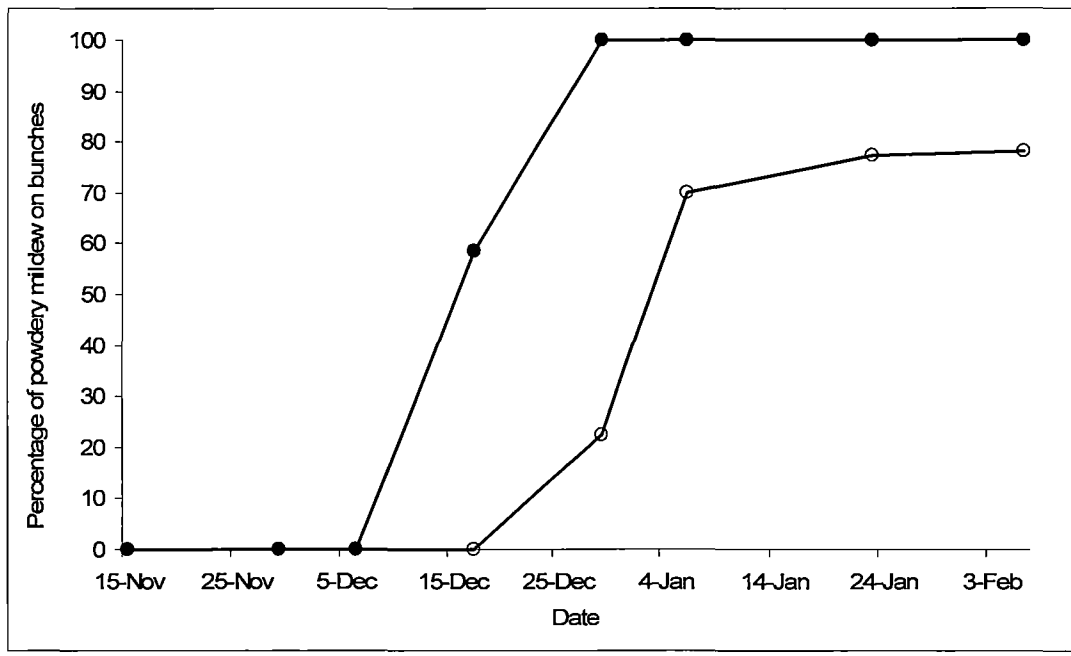


Figure 2.3 Mean incidence (●) and severity (○) of powdery mildew on Chardonnay bunches in non treated plots between December 6, 2005 (E-L 26, cap-fall complete) and February 6, 2006 (E-L 33, berries still hard and green) at Vineyard A.

Table 2.8 Powdery mildew incidence and severity on Chardonnay bunches at E-L 35 (veraison) at Vineyard A. Transformed data in parentheses. Means within columns for transformed data followed by the same letter are not significantly different at $P = 0.05$.

Treatment	Mean incidence (%)		Mean severity (%)	
Non-treated	100 (1.57)	b	77.3 (0.54)	b
ACE	27.0 (0.53)	a	0.34 (0.11)	a
ACE with FF[1] and LK[2]	13.0 (0.24)	a	0.05 (0.05)	a
Standard organic fungicide program for powdery mildew	31.0 (0.60)	a	0.17 (0.07)	a
lsd for transformed means	0.30		0.08	
df	12		12	
P	< 0.001		< 0.001	

[1] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v).
[2] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

2.4.3.3 *Suppression of botrytis bunch rot*

Sporulating *B. cinerea* was not evident on Chardonnay bunches at Vineyard A in 2005/06 and so bunches were assessed for latent *B. cinerea*. The incidence of latent *B. cinerea* was significantly greater in Chardonnay bunches from non-treated plots than from plots treated with ACE or the standard organic fungicide program for powdery mildew (Table 2.9). There was no significant difference among treatments for the severity of latent *B. cinerea* in Chardonnay bunches.

The percentage of berries at pre-bunch closure with latent *B. cinerea* in Riesling bunches at Vineyard B was < 2% and did not differ significantly among treatments (Table 2.10). Prior to harvest, there was a significantly greater incidence and severity of sporulating *B. cinerea* in the control treatments (plots treated with dechlorinated water or the standard fungicide program for powdery mildew) than in ACE treated

plots or bunches treated with the conventional spray program for both botrytis bunch rot and powdery mildew spray program (Table 2.10). By harvest, the incidence and severity of sporulating *B. cinerea* had declined as the weather remained sunny and dry.

Table 2.9 Effect of aerobic compost extracts (ACEs) on latent and sporulating (visible) *Botrytis cinerea*. At harvest in Vineyard A, 20 Chardonnay bunches per plot were surface sterilised and moist incubated to promote growth of latent *B. cinerea*. At pre-bunch closure in Vineyard B, prior to the application of treatments for this crop stage, latent *B. cinerea* was assessed by selecting 20 Riesling berries per 10 surface sterilised bunches per plot . The incidence and severity of sporulating *B. cinerea* on Riesling bunches (n=30) at E-L 36 (berries with intermediate °Brix values) was also assessed at Vineyard B. Arcsine-transformed data in parentheses. Means within columns for transformed data followed by the same letter are not significantly different at $P = 0.05$.

Treatment	Vineyard A			Vineyard B		
	Mean incidence of latent *B. cinerea* at harvest (%)	Mean severity of latent *B. cinerea* at harvest (%)	Percentage of 20 berries with sporulating latent *B. cinerea* at pre-bunch closure	Mean incidence of sporulating *B. cinerea* (%) at E-L 36	Mean severity of sporulating *B. cinerea* (%) at E-L 36	
Non-treated (Vineyard A) or dechlorinated water control (Vineyard B)	34 (0.62) b	2.61 (0.07)	0.25 (0.03)	13.3 (0.33) b	1.35 (0.03) b	
ACE	18 (0.43) a	3.80 (0.07)	0.83 (0.04)	9.44 (0.25) b	0.62 (0.06) b	
ACE with FF[1] and LK[2] (Vineyard A) or ACE with LK (Vineyard B)	18 (0.43) a	2.12 (0.05)	1.42 (0.10)	0.00 (0.00) a	0.00 (0.00) a	
Standard fungicide program for powdery mildew	13 (0.36) a	1.39 (0.03)	0.00 (0.00)	2.22 (0.08) a	0.12 (0.00) a	
Standard conventional fungicide program for powdery mildew and botrytis bunch rot	NA[3]	NA	0.25 (0.04)	1.11 (0.06) a	0.14 (0.00) a	
lsd for transformed means	0.18	0.05	0.07	0.18	0.03	
df	12	12	20	20	20	
P	0.046	0.321	0.089	0.004	0.003	

[1] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v).
[2] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).
[3] NA = not applied at this site.

2.4.3.4 ***Effect of compost extracts on grape yield, bunch compactness and juice composition***

In 2005/06, the mean total soluble solids of non-treated Chardonnay bunches at harvest was 21.8 °Brix, which was significantly greater than the ACE and standard organic treatments with 19.4 and 19.0 °Brix, respectively (lsd = 1.95, df = 12, P = 0.036). No other measures of juice composition or grape yield were taken at this site.

The average yield of Riesling vines across the trial site was below average at 7.1 tonne/ha. There were no significant differences in yield, average berry weight or components of juice quality across treatments (Table 2.11). The compactness of Riesling bunches varied across treatments with vines treated with ACE or the standard fungicide program having slightly but significantly higher percentage bunch openness than bunches treated with amended ACE or dechlorinated water (Table 2.11).

Table 2.11 Effect of aerobic compost extracts (ACEs) on yield, juice composition and bunch openness of Riesling vines at Vineyard B in 2006/07.

Treatment	Yield (tonne/ha)		Average berry weight (g)		Juice composition						Bunch openness (%)	
					pH		Titratable acidity		°Brix			
Dechlorinated water control	7.3	a	1.0	a	2.9	a	10.4	a	20.6	a	31.0	b
ACE	7.4	a	1.0	a	3.0	a	9.9	a	20.4	a	35.8	a
ACE with LK[1]	7.9	a	1.0	a	2.9	a	10.4	a	20.3	a	31.9	b
Standard conventional fungicide program for botrytis bunch rot and powdery mildew	7.2	a	1.1	a	2.9	a	10.2	a	20.6	a	34.7	a
lsd	5.02		0.45		0.09		1.06		0.54		2.83	
df	12		27		12		12		12		147	
P	0.992		0.888		0.528		0.703		0.562		0.003	

[1] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v)

2.4.3.5 *Microbial abundance in the phyllosphere*

Application of ACE increased the number of culturable bacteria, fungi and yeast on the leaf surface relative to pre-application levels at both vineyards (Table 2.12). The magnitude of the increase was approximately 100 fold at 1 h post-application. The number of culturable microorganisms on the leaf surface then declined gradually over 13 days, with the exception that the number of culturable bacteria at 13 days post-application on Riesling leaves was higher than observed on leaves at 1 day post-application. At 13 days post-application, the number of culturable microorganisms at both vineyards was higher than observed 1 h pre-application. At Vineyard B, 47 mm of rainfall was recorded 6 days after application of treatments.

Microbial numbers for all treatments 13 days post-application at Vineyard B are presented in Table 2.13. In summary, bacterial numbers, as measured by colony counts on NA, were not significantly different across treatments. When data for the dechlorinated water control were excluded from the analysis, ACE-treated leaves had significantly greater numbers of bacteria than the other two treatments (lsd = 0.14, df = 8, $P < 0.001$). The numbers of fungi and yeasts were significantly lower on leaves receiving the standard fungicide treatment when compared with all other treatments and the dechlorinated water control. Leaves treated with ACE amended with LK had significantly higher numbers of yeasts than leaves treated with ACE or dechlorinated water.

Table 2.12 The number of culturable bacteria, fungi and yeast quantified using different growth media, from leaves sampled at different times, before and after application of ACE at E-L 34 (veraison), to Chardonnay and Riesling vines at Vineyard A and Vineyard B, respectively.

Time of sampling, pre- or post-application of ACE	Bacteria (log_{10}cfu/ml)		Fungi (log_{10}cfu/ml)		Yeast (log_{10}cfu/ml)	
	Vineyard A	Vineyard B	Vineyard A	Vineyard B	Vineyard A	Vineyard B
1 h pre-application	4.90 d	4.65 e	4.72 d	4.23 c	4.62 e	4.43 c
1 h post-application	6.90 a	6.58 a	6.86 a	5.16 a	6.87 a	5.75 a
1 day post-applicatio	6.08 b	5.51 c	6.32 b	5.09 a	6.24 b	5.38 b
5 days post-application	6.00 b	5.29 d	5.99 b	4.76 b	5.98 c	5.07 b
13 days post-application	5.47 c	5.95 b	5.37 c	4.79 b	5.31 d	4.99 b
lsd	0.17	0.17	0.45	0.17	0.17	0.11
df	16	16	16	16	16	16
P	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Table 2.13 Number of culturable microorganisms (log_{10}cfu) on leaves measured at 13 days post-application of treatments applied at veraison to Riesling vines at Vineyard B.

Treatment	Number of microorganisms (log_{10}cfu/ml)					
	Bacteria		Fungi		Yeast	
Dechlorinated water control	4.20	a	4.84	a	4.94	b
ACE	4.39	a	5.29	b	4.44	b
ACE with LK[1]	4.18	a	4.18	a	5.60	a
Standard fungicide program for powdery mildew and botrytis bunch rot	4.23	a	3.83	c	3.79	c
lsd	0.23		0.12		0.17	
df	12		12		12	
P	0.23		< 0.001		< 0.001	

2.5 Discussion

Regular applications of ACE or amended ACE can suppress powdery mildew on grapevine fruit and foliage to commercially acceptable level in southern Tasmania. The severity of botrytis bunch rot in non-treated or water-treated plots at both field sites was not high enough to cause an economic impact. Nevertheless, ACE or amended ACE reduced the incidence of latent infection at Vineyard A and the incidence and severity of sporulation by *B. cinerea* at Vineyard B. This study is the first to illustrate suppression of two diverse pathogens following regular applications of ACE in the field environment. ACE was as effective as the standard commercial spray programs and there did not appear to be any additional benefit in amending ACE with the nutrients tested. Furthermore, application of ACE was not detrimental to juice quality (Brix°, TA, pH) or grape yield (Table 2.11), and has the added benefit of supplying beneficial nutrients to the crop canopy and reducing the cost of supplementary fertilisers. Based on this study, ACE has the potential to be incorporated into a disease management program for grapevine powdery mildew and botrytis bunch rot control in southern Tasmania. ACE should now be tested at more

sites and in a range of viticultural environments to confirm the potential for widespread application.

The risk of powdery mildew infection during the 2005/06 season was severe due to consistently humid conditions, lengthy periods of significant cloud coverage and high shoot vigour resulting from relatively high rainfall during spring. Each plot treated with ACE, amended ACE or the standard treatment received two sprays during the flowering and fruit set period, a time when developing fruit were highly susceptible to infection by *E. necator* (Ficke *et al.* 2002). These spray timings were probably critical in controlling powdery mildew on bunches given that the incidence of powdery mildew escalated on leaves and bunches in non-treated plots following flowering (Figs 2.2 and 2.3). In 2006/07, there were multiple rain events during veraison in mid February. ACE or the standard fungicide treatment prior to veraison may have reduced *B. cinerea* inoculum and/or latent infections in relation to control treatments at this time (Table 2.10). Differences in the amount of re-growth by *B. cinerea* after latency or the level of new berry infection in subsequent moist events during the ripening period might explain differences in disease expression between treatments.

Controlling powdery mildew on grape berries, even non-visible (diffuse) infections, is known to reduce the amount of bunch rotting fungi and wine spoilage yeasts on berries (Gadoury *et al.* 2007). In Vineyard A, the ACE treatment and the standard program for powdery mildew significantly reduced the incidence of bunch rot expressed after re-growth of latent *B. cinerea*, relative to the non-treated control. The powdery mildew fungicides and/or kelp meal used at this site either reduced potential berry damage by *E. necator*, and hence entry points for *B. cinerea*, and/or had direct

activity against *B. cinerea.* In Vineyard B, plots sprayed for powdery mildew control appeared to have a lower mean incidence and severity of sporulating *B. cinerea* than the dechlorinated water control, although the means were not separated statistically. A dechlorinated water control was included in the glasshouse experiment (for powdery mildew) and field trial with Riesling to determine whether water alone had an effect on the level of either powdery mildew or botrytis bunch rot but there was no evidence to support this hypothesis.

The results of the glasshouse experiment suggested that ACE had both protective and curative properties. Microscopy is required to observe the response of *E. necator* to ACE treatment before and after inoculation and to elucidate the mechanism of action (Crisp *et al.* 2006b). Potential mechanisms of action include hyperparasitism, or direct inhibition of spore germination or growth post germination by secretion of antimicrobial chemicals. Components of ACE might also have induced plant resistance to *E. necator* colonisation, given that ACE significantly reduced the severity of powdery mildew when applied up to 7 days prior to *E. necator* inoculation. Induced resistance in grapevine leaves to infection by *B. cinerea* was reported by Reglinski *et al.* (2005) after a chemical elicitor was applied 7 days prior to inoculation. This phenomenon could also be investigated in relation to powdery mildew by measuring the activity of pathogenesis-related proteins following ACE application (Aziz *et al.* 2003, Haggag and Saber 2007).

ACE augmented the number of fungi, bacteria and yeast on leaves during field experimentation (Table 2.12). The numbers of microorganisms declined from 1 h post-application to 13 days post-application reflecting the inability of all microorganisms from ACE to survive in a fluctuating environment. However, the

numbers of microorganisms were still above pre-application levels at 13 days post-application. The increase in the number of bacteria between 5 and 13 days post-application at Vineyard B might have been promoted by rainfall 6 days following ACE application. The persistence and growth of microorganisms used for single target biological control can be constrained by sub-optimal conditions of UV radiation, temperature, humidity, rainfall, nutrient availability and mechanism of action (Hurst *et al.* 2001). Unlike single biocontrol agents, ACE contains a diversity of microorganisms and it is possible that some taxa may survive under conditions in which other taxa would perish. In previous studies, a greater abundance of culturable microorganisms in ACE did not relate directly to pathogen suppression (Scheuerell and Mahaffee 2004, 2006, Chapter 1). Scheuerell and Mahaffee (2006), for example, found that addition of nutrients to ACE increased the number of bacteria but did not reduce grey mould on geranium in comparison with ACE with no nutrient amendments. At Vineyard B, there was a greater number of culturable yeasts 13 days after application of ACE amended with LK than the other treatments. However, this observation did not appear to be related to the disease suppressive ability of the extract. Nevertheless, leaves treated with ACE had significantly greater numbers of yeast than leaves treated with conventional fungicides and as a consequence bunches treated with ACE should be tested for the presence of 'wild' and spoilage yeasts prior to winemaking.

Both broad spectrum and single-target fungicides were used in the standard fungicide treatments. At E-L stage 34 (veraison), the number of culturable bacteria colonising the leaf surface at Vineyard B was not significantly different across treatments indicating that bacterial populations were relatively resistant to fungicide residues that had persisted from previous applications. Yeast and fungal populations,

however, were more sensitive to fungicide application. Sholberg *et al.* (2006) found similar results on grapevine leaves and berries with fungicide treatments reducing the number of fungi on berries and eliminating fungi on leaves.

Unlike previous studies, the amount of nutrients in ACE as well as the total quantity applied during the viticultural growing season is reported here. These measurements are particularly important because applications of nutrients can influence the susceptibility of vines to disease. Excessive amounts of nitrogenous fertilisers applied to soil can promote lush, green foliage suitable for the colonisation by obligate pathogens such as powdery mildew fungi, which obtain nutrition from living plant cells (Keller *et al.* 2003a). Dense canopies promoted by plant nutrition can also elevate relative humidity and promote infection by plant pathogens. In contrast, excessive amounts of potassium in soil can reduce the severity of powdery mildew and botrytis bunch rot in grapevines (Marschner 1986). Nutrients in ACE were applied to the foliage rather than the soil, but there was still the possibility that these nutrients altered plant physiology. The quantity of total nitrogen applied at Vineyard A (Table 2.6) was approximately three times greater than at Vineyard B, however suppression of powdery mildew was equivalent to the commercial treatments in both seasons. Furthermore, changes in crop yield or grape juice composition following season-long applications of ACE were not detected. There were also no obvious differences in shoot vigour among treatments, based on visual observation.

The presence of nutrients in ACE, especially nitrate, might influence its capacity to suppress leaf and fruit pathogens by supporting pathogenic microorganisms on the plant surface that require nutrients for spore germination. Welke (2004) reported a reduction in grey mould incidence and higher strawberry yields in comparison with

the control treatments, when aerated compost extracts based on cow manure with less than 1 mg/L of inorganic nitrogen (nitrite and nitrate) were sprayed on crops. Conversely, non-aerated compost extracts based on chicken manure with greater than 100 mg/L of nitrate and nitrite had little effect on crop yield or suppression of grey mould. A lack of nitrate in ACE, as illustrated at Vineyard A and in the cow manure compost in Welke (2004), demonstrates that nitrogen was mostly present in the organic form, including the microbial biomass. The presence of nitrate in ACE applied to plots at Vineyard B did not appear to affect its activity against *B. cinerea*, although the severity of botrytis bunch rot infection at this site was relatively low.

The electrical conductivity of ACE applied to grapevines in the field was, on average, $\geq$ 5 dS/m. Yield loss and phytotoxicity can occur when electrical conductivity in vineyard soils is above 7 dS/m (Stepphun *et al.* 2005). The conductivity level tolerated by foliar or fruit application of salt on grapevine does not appear to have been reported. The level of soluble salts in ACE did not appear to affect the yield of Riesling grapes in comparison with the control treatments (Table 2.11). Elevated salt levels may have contributed to the suppression of powdery mildew and botrytis bunch rot, but powdery mildew was also suppressed in the glasshouse experiment where the average conductivity was relatively low at 0.96 dS/m. Segarra *et al.* (2007) found that increasing the concentration of salt in compost reduced *B. cinerea* severity in cucumber plants and suggested that salt stress induced disease resistance in the host plant. Overall, these results suggest that the mechanism/s of ACE might vary in significance in different environments.

Further research is required to determine the effectiveness of ACE and its mechanism/s of action in a wide range of environments. Adoption of ACE as a

biological control agent will depend on strategies for integrating it into existing crop and disease management that allow the timing and number of applications of ACE to be optimised in relation to pathogen activity and canopy susceptibility to disease. Knowledge of the mechanism of ACE in suppressing these grapevine pathogens will aid development of the disease management strategy.

CHAPTER 3:

POTENTIAL FOR GROWTH OF HUMAN PATHOGENIC BACTERIA IN AEROBIC COMPOST EXTRACTS (ACE)

3.1 Abstract

Aerobic compost extract (ACE) is a watery extract of compost and a type of compost tea that is applied by horticultural producers to fruit and foliage of crops for improved plant health and crop protection. Commercial open windrow compost was collected during the cooling phase of composting when the internal windrow temperature was 50°C and the presence of *Escherichia coli*, *Listeria monocytogenes* and *Bacillus cereus* was assessed. There was a negligible number of human pathogenic bacteria in compost samples, so aerobic compost extracts (ACEs) with a ratio of 1 part compost to 3 parts water were prepared and inoculated with non-pathogenic streptomycin resistant *E. coli* (1 x 10^7 cfu/ml), which were then enumerated over a 72 h period of extraction. There was no significant change in the number of streptomycin resistant *E. coli* during extraction of ACE that has not been amended with nutrients. However, there was a significant increase in *E. coli* numbers when 0.8% fish hydrolysate or 1% molasses were introduced to ACE 24 h after extraction commenced. Introduction of 0.5-2% liquid kelp or a mixture of 1.7% liquid kelp and 0.8% fish hydrolysate led to a decline in the number of *E. coli*. Increasing the liquid kelp concentration to 8% in the mixture with 0.8% fish hydrolysate resulted in *E. coli* numbers equivalent to ACE amended with 0.8% fish hydrolysate only.

There appeared to be no relationship between the number of *E. coli* and the abundance of culturable bacteria and fungi in ACEs amended with nutrients,

although a low oxygen concentration, pH and high conductivity was associated with increased *E. coli* numbers in an ACE amended with 1% molasses. Fermentative metabolism of the simple carbohydrates in molasses might explain this increase in *E. coli* numbers and the reduction in pH. The results of this study imply that methods should be identified and imposed to assure that human pathogens do not contaminate ACEs during or after preparation.

3.2 Introduction

Agricultural producers expect commercial sources of compost or amended compost to be free of human and plant pathogens. In Australia, the quality and application of compost is generally not regulated by the government, even though application of the Australian Standard for production of compost, soil conditioners and mulches (AS 4454 2003) is required by law. This Standard requires that commercially prepared composts have achieved conditions equivalent to pasteurisation for elimination of vegetative human pathogens. Compost analyses and audits made by request to a state department of health are voluntary (Anon. 2004) and this situation can lead to a quandary, with regard to product safety, for producers, farm workers and the consumer when results are not available.

Aerobic compost extracts are applied to fruit, foliage and/or soil to enhance crop yield and suppress diseases caused by plant-pathogenic fungi and bacteria. In brief, aerobic compost extract (ACE) is prepared by suspending a porous bag or sieve containing aerobic compost in oxygenated water for 24 to 72 h. ACE, a type of compost "tea", is being used widely by a small sector of horticultural producers (Touart 2000, Scheuerell and Mahaffee 2002). However, retailers of fresh food and consumers are increasingly concerned about the possibility of human pathogens in

poor quality compost applied to horticultural crops. An additional concern is re-establishment and growth of human pathogens in ACE, especially when nutrients are added during extract production (Duffy *et al.* 2004).

Few scientists have published research on human pathogen presence and growth in ACE prepared from mature, commercially available composts. Results from *in vitro* assays involving inoculation of unamended ACE with *E. coli* and/or *S. enterica* revealed no significant increase in populations of these human pathogens (Duffy *et al.* 2004, Kannangara *et al.* 2006). Nevertheless, these studies revealed a strong positive correlation between molasses and kelp concentrations in ACE amended with these nutrients and *E. coli* numbers. Moreover, *E. coli* and *S. enterica* populations can proliferate in water-based solutions of fish hydrolysate, kelp, seaweed and/or humic acids, in the absence of ACE (Ingram and Millner 2007). To ascertain whether or not results from *in vitro* studies applied to commercial production conditions, Ingram and Millner (2007) inoculated ACE or ACE amended with nutrients with human pathogens, using extracts prepared by a commercial compost tea brewer. There was a significant increase in the numbers of *E. coli*, *S. enterica* and total fecal coliforms in treatments supplemented with a mixture of molasses, bat guano, sea bird guano, powdered soluble kelp, citric acid, Epsom salts, ancient seabed minerals and calcium carbonate or a mixture of powdered soluble kelp, liquid humic acids and rock dust. These results suggest that practitioners adopting ACE need to be especially cautious when adding nutrients to compost extracts if human pathogens contaminate ACE during production and if conditions during or after production enable pathogens to grow and multiply.

The results in Chapter 1 illustrated ACE production conditions necessary for consistent suppression of the fungal plant pathogen *Botrytis cinerea* after inoculation of detached bean leaflets that had been treated with a test batch of ACE. The most pathogen-suppressive ACE identified in Chapter 1 had the greatest diversity of bacteria and fungi and was prepared from compost collected from open windrows in the cooling phase of composting, at an internal windrow temperature between 40°C and 50°C. These production conditions for ACE were standardised and adopted for further testing. During the viticultural seasons of 2005/06 and 2006/07 (Chapter 2), application of standardised ACE to commercial wine grapes demonstrated reduction of powdery mildew, caused by *Erysiphe necator*, to commercially acceptable levels on Chardonnay grape clusters and Riesling leaves. The next step in the research was to determine the safety of standardised ACE for use in horticultural production.

This study in relation to the safety of ACE addressed three objectives. The first objective was to investigate the presence of human pathogenic bacteria in commercially produced composts during the early secondary mesophilic stage of composting. The second objective was to determine, using a non-pathogenic, streptomycin-resistant *E. coli,* the potential for populations of *E. coli* to multiply in ACE or ACE amended with nutrients, prepared under small-scale laboratory conditions that simulated standardised production. The final objective was to investigate if any increase in the number of *E. coli,* inoculated into a particular ACE, also altered the total number of culturable bacteria and/or fungi when compared with an ACE that did not support the multiplication of the test strain of *E. coli*. The use of compost from the early mesophilic stage of composting differentiates this research from previous studies that utilised mature compost and the results support earlier

reports that adding particular nutrients to compost extracts can lead to re-growth of human pathogenic bacteria.

3.3 Materials and Methods

3.3.1 Compost production

Compost was produced by Soil First Pty Ltd (Parrata, Tasmania, Australia) as described in Chapter 1. Briefly, compost was initiated in open windrows with an initial C:N of 30:1. The primary raw ingredients included mulched green waste from municipal councils in Tasmania and waste from Atlantic salmon (*Salmo salar*) aquaculture. A 10 kg and a 300 g sample of compost were collected at a depth of 1 m at three positions along a windrow: approximately 10 m from each end of the 50 m windrow and in the centre of the windrow, when the internal temperature was approximately 50°C in the cooling phase of composting. The three 10 kg samples were placed in containers that had been surface sterilised with 70% ethanol and transported to the New Town Research Laboratories for pilot-scale extraction. The three 300 g samples were placed in autoclaved containers and transported directly to the microbiology laboratory of the University of Tasmania for laboratory-scale extraction. Both laboratories are located in Hobart, approximately 1 h by road from Parrata.

3.3.2 Production of ACE

Three 100 L tanks were surface sterilised with 70% ethanol and ACE was prepared as described in Chapter 1 with a compost to water ratio of 1:3. Prior to compost addition (0 h extraction time) and after every 24 h of extraction, up to 72 h, 500 ml samples of ACE were aseptically collected from each tank. The samples were placed in an icebox and transported 15 min by road to the microbiology laboratory for

challenge trials with a non-pathogenic streptomycin resistant mutant of *E. coli* M23. In addition, a sample of 48 h ACE was sent to the Environmental Analysis Laboratory (Lismore, NSW, Australia) for analysis of macro-nutrients.

3.3.3 Analysis of human pathogens

A membrane filtration method was used to test compost and ACE samples for presence of *E. coli*, *L. monocytogenes* and *B. cereus*. For each 300 g compost sample, 100 g of compost and 200 ml of sterile distilled water were placed in a Stomacher® Classic filter bag (177 mm x 304 mm, Seward Laboratories, UK) with a 25 µm inner mesh. The liquid that was separated from the compost particles was passed through a 47 mm-diameter sterile vacuum filter (Sterifil® Aseptic System, Millipore Co.) containing a sterile membrane filter paper with 0.45 µm diameter pores (Whatman Microplus – 21ST white/black). After filtration, each filter paper was placed grid side up onto one of three selective agar media in 90 mm-diameter Petri plates and prepared according to the manufacturers' instructions: Membrane Lauryl Sulfate broth (MLS; Oxoid CM0451; Oxoid Australia Limited) solidified with 1.5% agar for *E. coli*, Listeria selective agar (Oxford formulation; Oxoid CM0856 with SR0140 Supplement, Oxoid Australia Limited) for *Listeria* sp. and Bacillus selective agar for *Bacillus* sp. (Oxoid CM0617 with SR0099 Supplement, Oxoid Australia Limited). The same filtration and plating procedure was completed with 500 ml of each ACE prepared at New Town Research Laboratories. MLS plates were inverted and incubated at 30°C for 4 h and then at 44°C for 14 h. Both Bacillus selective agar and Listeria selective agar plates were inverted and incubated at 37°C for 24 h.

Colonies of bacteria were picked off and, as appropriate, subcultured for further identification. Beta-haemolysis on Sheep blood agar (Oxoid CM0271, Oxoid,

Australia Limited) and microscopy were used to confirm presumptive *B. cereus*. Confirmation of presumptive *Listeria* sp. was based on beta-haemolysis and presumptive *E. coli* were confirmed using the API 20E system (Biomerieux Inc., Baulkham, NSW, Australia).

3.3.4 Persistence and multiplication of *E. coli* in ACE or ACE amended with nutrients

3.3.4.1 *Laboratory scale ACE production and inoculation conditions*

To compare the potential for populations of *E. coli* to persist and/or multiply in ACE or amended ACE, standardised ACE was prepared on a small scale in the laboratory. Sterile flasks of 2 L capacity were filled with 1 L of sterile distilled water. A pond pump (Bianco, BIAWFP200, 200 L/h, 240 v, White International Pty Ltd, Milperra, Australia) sterilised with 70% ethanol was placed in each flask to ensure a dissolved oxygen level above 6 mg/L. An autoclaved, 100 % polypropylene bag (6 cm x 25 cm) was filled with 300 g of compost and suspended in the flask above the pond pump.

At 0 h extraction time, immediately prior to addition of bags of compost, three flasks per experimental treatment, representing compost from three windrow positions, were inoculated with a non-pathogenic streptomycin resistant strain of *E. coli*, M23, to a final concentration of 1×10^7 colony forming units (cfu)/ml. *E. coli* strain M23 was obtained from the culture collection of the School of Agricultural Science, University of Tasmania, Tasmania, Australia and was originally isolated from *E. coli* M23 by culture on nutrient agar including 100 mg/ml of streptomycin and selection of colonies. Three flasks were not inoculated and acted as the control treatment. One

or more nutrients, described below, were added to each of three flasks 24 h after extraction commenced.

Electrical conductivity, dissolved oxygen, pH, temperature, and nitrate were measured immediately prior to extraction and every 24 h, up to 72 h. Nitrate was determined using nitrate Merckoquant® strip tests (Merck Pty Ltd). The other physical parameters were measured using WTW Handheld 340i or Inolab meters (Merck Pty Ltd).

3.3.4.2 *Experiment one: effect of nutrient additives on E. coli populations*

A total of 18 flasks (three flasks per treatment) were prepared, as described in the previous section, for the following treatments: non-inoculated ACE, inoculated ACE, inoculated ACE amended with molasses (M), Liquid Kelp™ (LK), Foundation Fish™ (FF) hydrolysate or a mixture of LK and FF. These nutrients and the concentration applied in treatments are described in Table 3.1.

Table 3.1 Raw ingredients and quantity of each product added to ACE after 24 h of extraction.

Product	Raw ingredients in product	Quantity
Molasses (Roberts Pty Ltd, Tasmania)	Molasses	1 part M:100 parts ACE (v/v) or 1%
Foundation Fish™ (FF) (Soil First Pty Ltd, Tasmania)	hydrolysate of atlantic salmon (*Salmo salar*) aquacultural waste, post filleting	1 part FF:120 parts ACE (v/v) or 0.8%
Liquid Kelp™ (LK) (Soil First Pty Ltd, Tasmania)	Bull kelp (*Durvillaea potatorum*)	1 part LK:60 parts ACE (v/v) or 1.7%

3.3.4.3 *Experiments two and three: effect of liquid kelp and/or fish hydrolysate on E. coli populations*

The effect of adding various quantities of LK to ACE was investigated. Twenty-one flasks were prepared for the following treatments: a non-inoculated control, inoculated ACE, and inoculated ACE amended with 0.5%, 1%, 2%, 4% or 8% (v/v) LK. LK was included 24 h after extraction commenced and three flasks were utilised per treatment. This experiment was repeated with the exception that 0.83% (v/v) FF was added to each flask at 24 h.

3.3.4.4 *Enumeration of microorganisms in all experiments*

At 72 h, 10 ml was sampled from each flask to enumerate culturable, aerobic, bacteria and fungi. Serial dilutions were prepared using sterile 0.14 M sodium chloride. A sample of 100 µl of each dilution was plated, in triplicate, on nutrient agar and acidified potato dextrose agar (APDA), respectively. Nutrient agar plates were inverted and incubated at 25°C for 72 h and APDA plates were incubated in the

dark at 21°C ± 4°C for 5 days. Following incubation, colonies were enumerated to calculate cfu/ml in each ACE sampled.

Samples of ACE were collected from each flask at 0, 24, 48 and 72 h extraction and *E. coli* was enumerated by spreading 100 µl of appropriate dilutions on nutrient agar plates containing 100 mg filter sterilised streptomycin per ml of nutrient agar. At 24 h extraction the ACE samples were collected immediately prior to nutrient amendment. The plates were inverted and incubated at 37°C for 24 h prior to enumeration of colonies.

3.3.5 Data analyses

Analysis of variance (ANOVA) in Genstat™ for Windows 8th Edition was applied to treatments and cfu/ml was transformed to $\log_{10}$cfu/ml, as appropriate, to homogenise the variance in the data.

3.4 Results

3.4.1 Nutrient content and physical characters of ACE and amended ACE

A sample of 48 h ACE from pilot-scale production (10 kg of compost in 30 L water) contained 185, 25.3 and 620 mg/L of the major nutrients N, P and K, respectively, i.e. a ratio of approximately 7:1:24. It also contained 57 mg/L of calcium, 43 mg/L of magnesium and 992 mg/L of chloride.

The mean physical characters of compost extracts from laboratory-scale production (1 L water) are presented in Tables 3.2A, B, C and D. In summary, at 24, 48 and 72 h extraction time, the mean pH of ACE or ACE amended with one or more nutrients was close to neutral across almost all experiments, with the exception of ACE

amended with M, which was more acidic at 48 and 72 h than other ACEs prepared in experiment one. The amount of dissolved oxygen in all ACEs in experiment one was above 6 mg/L, excluding ACE amended with FF and ACE amended with M at 48 and 72 h extraction time (Table 3.2A). When combining data from 24, 48 and 72h extraction times; there was significantly less dissolved oxygen (3.4 mg/L) in ACE amended with M than other ACEs prepared in experiment one (Table 3.2D). . In experiment two and three the amount of dissolved oxygen declined over extraction time for all treatments (Table 3.2B, C). The mean conductivity of water used to prepare ACE was less than 1 dS/m and conductivity increased with extraction time. ACE amended with FF had a significantly higher mean conductivity than unamended ACE in experiment one (Table 3.2D). The mean temperature of all ACEs was between 22°C and 26°C (Table 3.2A, B, C, D) and there was no significant difference in mean temperature among treatments in experiments one, two or three when 24, 48 and 72h extraction times were combined for analysis (Table 3.2D).. Nitrate was present in all extracts excluding 0 h extraction time. There was no significant difference among extracts for nitrate concentration in experiments one, two or three when 24, 48 and 72h extraction times were combined for analysis (Table 3.2D).

3.4.2 Analysis of human pathogens in compost and unamended ACE

None of the three composts sampled contained detectable levels of *E. coli*, *L. monocytogenes* or *B. cereus*. Similarly, none of the ACEs prepared from compost with an internal windrow temperature of 50°C and at various windrow positions contained detectable levels of those pathogens.

3.4.3 Multiplication of *E. coli* in ACE and ACE amended with nutrients

The number of streptomycin resistant *E. coli* in unamended ACE was similar across all extraction times (0 to 72 h) for experiment one (Table 3.3). In contrast, in experiment two there was a slightly significant decline in *E. coli* numbers from 0 to 72 h extraction time (Table 3.3). Numbers of *E. coli* were not significantly different in any treatments in all three experiments 24 h after extraction commenced and before amendment of specific treatments with nutrients (Figs 3.1, 3.2 and 3.3). At 48 and 72 h extraction time, there were significant differences between unamended ACE and treatments where ACE was amended with one or more nutrients (Fig. 3.1). There was a steady increase in *E. coli* numbers between 24 and 72 h in ACE amended with 1% M or 0.8% FF in comparison with unamended ACE. In contrast, there was a decline in *E. coli* numbers between 24 and 72 h when 1.7% LK was added to ACE. There was a sharp decline in *E. coli* numbers in ACE amended with a combination of LK and FF from 48 to 72 h.

There was no significant difference among treatment means for the number of culturable bacteria when ACE was amended with nutrients (Table 3.4). In contrast, there was a significant increase in numbers of fungi in ACE amended with 1.7% LK or 1% M when compared with all other treatments, including unamended ACE (Table 3.4).

3.4.3.1 *Effect of liquid kelp and/or fish hydrolysate on E. coli populations*

The number of *E. coli* in unamended ACE appeared to increase between 48 h and 72 h extraction time, but the number was still less than observed at 0 and 24 h (Fig. 3.2). At 48 h extraction time, there were significantly fewer *E. coli* in ACE with 1% LK in comparison with unamended ACE (Fig. 3.2). At 72 h extraction time, all LK treatments reduced the number of *E. coli*, with the greatest reduction in *E. coli*

numbers at 0.5%, 1% or 2% LK. The number of culturable bacteria was not altered significantly by the addition of 0.5% to 8% LK to ACE (Table 3.4). In relation to unamended ACE, there were significantly fewer fungi when ACE was amended with 4% or 8% LK, yet significantly more fungi when ACE was amended with 1% LK. In short, the number of fungi appeared to increase as the concentration of LK approached 1% and then declined as the concentration of LK increased beyond 1%.

Table 3.2A Mean (± standard deviations) pH, dissolved oxygen, conductivity, temperature and nitrate in ACE and ACE with amendments for experiment one.

Treatment	Extraction time (h)	pH	Dissolved oxygen (mg/L)	Conductivity (mS/cm)	Temperature (°C)	Nitrate (mg/L)
Unamended ACE	0	7.13± 0.10	9.42 ± 0.64	0.08 ± 0.00	22.4 ± 0.29	0.00 ± 0.00
	24	7.26 ± 0.32	9.49 ± 0.12	2.85 ± 0.37	23.1 ± 0.95	250 ± 0.00
	48	7.38 ± 0.05	10.1 ± 0.56	3.48 ± 0.22	23.6 ± 0.3	333 ± 144
	72	7.28 ± 0.03	9.75 ± 0.32	3.18 ± 0.53	23.8 ± 0.25	333 ± 144
ACE + LK	0	7.10 ± 0.09	9.85 ± 0.20	0.08 ± 0.02	22.2 ± 0.36	0.00 ± 0.00
	24	7.03 ± 0.07	9.53 ± 0.08	3.00 ± 0.28	23.6 ± 0.2	150 ± 86.6
	48	7.18 ± 0.03	9.78 ± 0.14	3.20 ± 0.06	23.5 ± 0.31	200 ± 86.6
	72	7.2 ± 0.02	9.66 ± 0.13	3.21 ± 0.05	23.3 ± 0.27	200 ± 86.6
ACE + FF	0	7.14 ± 0.14	9.70 ± 0.04	0.08 ± 0.00	22.6 ± 0.06	0.00 ± 0.00
	24	7.04 ± 0.07	9.34 ± 0.21	2.71 ± 0.32	23.5 ± 0.55	200 ± 86.6
	48	6.52 ± 0.04	5.71 ± 0.18	4.80 ± 0.19	23.5 ± 0.31	250 ± 0.00
	72	6.77 ± 0.22	5.45 ± 0.21	4.84 ± 0.25	23.7 ± 0.15	250 ± 0.00
ACE + LK + FF	0	7.24 ± 0.27	9.71 ± 0.12	0.08 ± 0.00	22.2 ± 0.55	0.00 ± 0.00
	24	7.09 ± 0.08	9.48 ± 0.20	2.74 ± 0.19	23.9 ± 0.32	150 ± 86.6
	48	6.60 ± 0.11	7.77 ± 0.14	7.77 ± 0.14	25.1± 0.08	250 ± 0.00
	72	6.47 ± 0.30	7.78 ± 0.15	5.13 ± 0.12	23.6 ± 0.45	200 ± 86.6
ACE + M	0	7.08 ± 0.09	9.74 ± 0.11	0.09 ± 0.00	22.8 ± 0.1	0.00 ± 0.00
	24	7.24± 0.10	9.47 ± 0.22	3.03 ± 0.10	23.6 ± 0.15	200 ± 86.6
	48	5.20 ± 0.02	0.42 ± 0.24	5.49 ± 0.21	23.5 ± 0.40	250 ± 0.00
	72	5.11 ± 0.10	0.34 ± 0.16	5.53 ± 0.22	23.4 ± 0.21	250 ± 0.00

[1] Molasses added after 24 h of extraction at a concentration of 1 part M:100 parts ACE (v/v).
[2] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v).
[3] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

Table 3.2B Mean (± standard deviations) pH, dissolved oxygen, conductivity, temperature and nitrate in ACE and ACE with amendments for experiment two.

Treatment	Extraction time (h)	pH	Dissolved oxygen (mg/L)	Conductivity (dS/m)	Temperature (°C)	Nitrate (mg/L)
Unamended ACE	0	7.09± 0.07	8.47 ± 0.44	0.67 ± 0.47	22.7 ± 0.40	0.00 ± 0.00
	24	7.40 ± 0.25	7.57 ± 0.34	8.29 ± 0.07	22.6 ± 0.15	333 ± 144
	48	7.46 ± 0.18	5.03 ± 0.23	11.4 ± 1.28	23.6 ± 0.23	333 ± 144
	72	7.49 ± 0.12	5.28 ± 0.35	10.6 ± 0.45	23.7 ± 0.06	333 ± 144
ACE + 0.5% LK[1]	0	7.08 ± 0.03	8.40 ± 0.15	0.89 ± 0.11	22.7 ± 0.10	0.00 ± 0.00
	24	7.36 ± 0.42	7.76 ± 0.08	8.26 ± 0.04	22.6 ± 0.06	333 ± 144
	48	7.33 ± 0.19	5.55 ± 0.35	10.6 ± 1.44	23.5 ± 0.40	333 ± 144
	72	7.25 ± 0.07	5.83 ± 0.19	10.5 ± 0.61	23.6 ± 0.23	333 ± 144
ACE + 1% LK	0	7.09 ± 0.10	8.28 ± 0.06	0.72 ± 0.30	22.2 ± 0.12	0.00 ± 0.00
	24	7.28 ± 0.09	7.65 ± 0.05	8.20 ± 0.11	22.9 ± 0.15	250 ± 0.00
	48	7.38 ± 0.14	5.44 ± 0.38	11.2 ± 1.10	23.3 ± 0.17	250 ± 0.00
	72	7.35 ± 0.28	5.72 ± 0.36	10.5 ± 0.44	23.5 ± 0.23	250 ± 0.00
ACE + 2% LK	0	7.08 ± 0.08	8.33 ± 0.10	0.44 ± 0.42	22.5 ± 0.52	0.00 ± 0.00
	24	7.49 ± 0.19	7.63 ± 0.27	8.13 ± 0.17	22.7 ± 0.12	250 ± 0.00
	48	7.37 ± 0.16	5.15 ± 0.75	11.3 ± 1.85	23.4 ± 0.26	250 ± 0.00
	72	7.35 ± 0.07	5.61 ± 0.40	9.99 ± 0.32	23.7 ± 0.10	250 ± 0.00
ACE + 4% LK	0	7.28 ± 0.33	8.25 ± 0.07	0.43 ± 0.49	22.4 ± 0.10	0.00 ± 0.00
	24	7.53 ± 0.16	7.63 ± 0.41	8.08 ± 0.12	22.9 ± 0.21	333 ± 144
	48	7.41 ± 0.10	5.75 ± 0.51	13.8 ± 1.62	23.6 ± 0.10	250 ± 0.00
	72	7.39 ± 0.25	5.18 ± 0.06	10.0 ± 0.31	23.4 ± 0.40	250 ± 0.00
ACE + 8% LK	0	7.07 ± 0.06	8.10 ± 0.19	0.93 ± 0.02	22.4 ± 0.10	0.00 ± 0.00
	24	7.30 ± 0.28	7.72 ± 0.39	8.10 ± 0.03	22.3 ± 0.27	333 ± 144
	48	7.48 ± 0.06	5.22 ± 0.18	12.1 ± 1.87	23.3 ± 0.21	500 ± 0.00
	72	7.42 ± 0.33	5.28 ± 0.06	10.0 ± 0.07	23.3 ± 0.25	500 ± 0.00

[1]Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

Table 3.2C Mean (± standard deviations) pH, dissolved oxygen, conductivity, temperature and nitrate in ACE and ACE with amendments for experiment three.

Treatment	Extraction time (h)	pH	Dissolved oxygen (mg/L)	Conductivity (mS/cm)	Temperature (°C)	Nitrate (mg/L)
Unamended ACE + FF[1]	0	7.12 ± 0.03	8.47 ± 0.44	0.67 ± 0.47	22.7 ± 0.40	0.00 ± 0.00
	24	7.10 ± 0.09	7.57 ± 0.34	8.29 ± 0.07	22.6 ± 0.15	333 ± 144
	48	7.31 ± 0.35	5.03 ± 0.23	11.4 ± 11.3	23.6 ± 0.25	333 ± 144
	72	7.27 ± 0.30	5.27 ± 0.35	10.6 ± 0.45	23.7 ± 0.06	333 ± 144
ACE + 0.5% LK[2] + FF	0	7.10 ± 0.03	8.40 ± 0.15	0.89 ± 0.11	22.7 ± 0.10	0.00 ± 0.00
	24	7.07 ± 0.07	7.79 ± 0.08	8.26 ± 0.04	22.6 ± 0.06	333 ± 144
	48	7.20 ± 0.36	5.55 ± 0.35	10.6 ± 1.44	23.5 ± 0.40	333 ± 144
	72	6.99 ± 0.02	5.83 ± 0.19	10.5 ± 0.61	23.6 ± 0.23	333 ± 144
ACE + 1% LK + FF	0	7.10 ± 0.08	8.28 ± 0.06	0.72 ± 0.30	22.2 ± 0.12	0.00 ± 0.00
	24	6.99 ± 0.10	7.65 ±0.05	8.20 ± 0.11	22.9 ± 0.15	250 ± 0.00
	48	6.75 ± 0.24	5.44 ± 0.38	11.2 ± 1.10	23.3 ± 0.17	250 ± 0.00
	72	6.94 ± 0.69	5.72 ± 0.36	10.5 ± 0.44	23.5 ± 0.23	250 ± 0.00
ACE + 2% LK + FF	0	7.33 ± 0.43	8.33 ± 0.10	0.44 ± 0.42	22.5 ± 0.52	0.00 ± 0.00
	24	6.93 ± 0.05	7.63 ± 0.27	8.13 ± 0.17	22.7 ± 0.12	250 ± 0.00
	48	6.78 ± 0.49	5.15 ± 0.75	11.3 ± 1.85	23.4 ± 0.27	250 ± 0.00
	72	6.74 ± 0.35	5.61 ± 0.40	9.99 ± 0.32	23.7 ± 0.10	250 ± 0.00
ACE + 4% LK + FF	0	7.05 ± 0.11	8.25 ± 0.07	0.43 ± 0.49	22.4 ± 0.10	0.00 ± 0.00
	24	6.83 ± 0.12	7.63 ± 0.41	8.08 ± 0.12	22.9 ± 0.21	333 ± 144
	48	6.88 ± 0.31	5.75 ± 0.51	13.8 ± 1.62	23.4 ± 0.10	250 ± 0.00
	72	6.94 ± 0.11	5.18 ± 0.06	10.0 ± 0.32	23.4 ± 0.40	250 ± 0.00
ACE + 8% LK + FF	0	7.12 ± 0.01	8.10 ± 0.19	0.93 ± 0.02	22.4 ± 0.10	0.00 ± 0.00
	24	6.48 ± 0.35	7.72 ± 0.39	8.10 ± 0.03	22.3 ± 0.27	333 ± 144
	48	6.72 ± 0.47	5.22 ± 0.18	12.1 ± 1.87	23.3 ± 0.21	500 ± 0.00
	72	6.99 ± 0.18	5.28 ± 0.06	10.0 ± 0.07	23.3 ± 0.25	500 ± 0.00

[1] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v).
[2] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

Table 3.2D. Mean pH, dissolved oxygen, conductivity, temperature and nitrate in ACE and ACE with amendments for data combined from extraction times 24, 48 and 72 h.. Although the means for variables measured at 24 h were different from those measured at 48 and 72 h in some treatments of each experiment (Tables 3.4 A, B and C), data were combined from these three extraction times for ANOVA and statistical separation of relatively large differences among means within an experiment. Means for compost extraction time were from data combined from all human pathogen inoculation experiments. Treatments with the same letter are not significantly different at $P = 0.05$. The least significant difference is specified as 'lsd' and 'ns' means 'not significant'.

Experiment	Treatment	pH	Dissolved oxygen (mg/L)	Conductivity (mS/cm)	Temperature (°C)	Nitrate (mg/L)
One	Unamended ACE	7.4 a	8.4 b	3.2 c	23	306
	ACE + 1% M[1]	5.9 d	3.4 d	4.7 b	24	233
	ACE + 1.7% LK[2]	7.1 b	9.7 a	3.1 c	24	183
	ACE + 0.8% FF[3]	6.8 c	6.8 c	7.8 a	24	233
	ACE + 1.7% LK + 0.8% FF	6.7 c	8.3 b	7.6 a	24	200
	lsd	0.15	0.35	0.63	ns	ns
Two	Unamended ACE	7.5	7.7 a	5.1 c	23	333
	ACE + 0.5% LK	7.3	7.3 ab	6.1 a	23	333
	ACE + 1% LK	7.3	7.6 ab	5.4 b	23	250
	ACE + 2% LK	7.4	7.2 b	6.1 a	23	250
	ACE + 4% LK	7.4	7.7 a	5.5 b	23	277
	ACE + 8% LK	7.4	7.3 ab	6.3 a	23	444
	lsd	ns	0.44	0.25	ns	ns
Three	ACE + 0% LK + 0.8% FF	7.2 a	5.6 b	10.1	25	250
	ACE + 0.5% LK + 0.8% FF	7.1 a	6.4 a	9.8	25	250
	ACE + 1% LK + 0.8% FF	6.9 b	6.3 a	10.0	25	250
	ACE + 2% LK + 0.8% FF	6.8 bc	6.1 ab	9.8	25	250
	ACE + 4% LK + 0.8% FF	6.9 b	6.2 a	10.7	25	333
	ACE + 8% LK + 0.8% FF	6.7 c	6.1 ab	10.1	25	333
	lsd	0.10	0.55	ns	ns	ns
Extraction time (h)	0	7.1	8.9 a	0.3	23	0.0
	24	7.1	7.1 b	6.3	24	252
	48	7.0	6.2 c	8.2	23	276
	72	7.0	6.0 c	7.6	24	303
	lsd	ns	0.60	0.57	ns	26

[1] Molasses added after 24 h of extraction at a concentration of 1 part M:100 parts ACE (v/v). [2] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v). [3] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

Table 3.3 Effect of extraction time on the mean number of streptomycin-resistant *Escherichia coli* M23 in unamended ACE. Treatments with the same letter are not significantly different at $P = 0.05$. The least significant difference is specified as 'lsd' and 'ns' means 'not significant'.

Extraction time (h)	Mean number of *E. coli* (log_{10}cfu/ml)	
	Experiment one	Experiment two
0	7.26	6.92 a
24	7.06	6.79 b
48	7.26	6.08 d
72	7.07	6.55 c
lsd	ns	0.12

Table 3.4 Effect of ACE and ACE with amendments on the mean number of culturable bacteria and fungi at 72 h extraction time (log_{10}cfu/ml). Treatments followed by the same letter are not significantly ($P < 0.05$). ns = not significantly different.

Experiment	Treatment	Mean number of bacteria (log_{10}cfu/ml)	Mean number of fungi (log_{10}cfu/ml)
One	Unamended ACE	9.39	7.46 b
	ACE + 1% M[1]	9.77	8.66 a
	ACE + 1.7% LK[2]	9.20	8.77 a
	ACE + 0.8% FF[3]	9.93	7.94 b
	ACE + 1.7% LK + 0.8% FF	9.33	7.80 b
	lsd	ns	0.68
Two	Unamended ACE	9.30	7.59 b
	ACE + 0.5% LK	9.10	7.73 ab
	ACE + 1% LK	9.16	7.96 a
	ACE + 2% LK	9.20	7.85 ab
	ACE + 4% LK	9.26	7.01 c
	ACE + 8% LK	9.24	7.09 c
	lsd	ns	0.33
Three	ACE + 0% LK + 0.8% FF	9.42	7.58 b
	ACE + 0.5% LK + 0.8% FF	9.25	8.39 a
	ACE + 1% LK + 0.8% FF	9.22	8.39 a
	ACE + 2% LK + 0.8% FF	9.25	8.46 a
	ACE + 4% LK + 0.8% FF	9.23	8.56 a
	ACE + 8% LK + 0.8% FF	9.32	8.59 a
	lsd	ns	0.35

[1] Molasses added after 24 h of extraction at a concentration of 1 part M:100 parts ACE (v/v).
[2] Foundation Fish™ hydrolysate added after 24 h of extraction at a concentration of 1 part FF:120 parts ACE (v/v).
[3] Liquid Kelp™ added after 24 h of extraction at a concentration of 1 part LK:60 parts ACE (v/v).

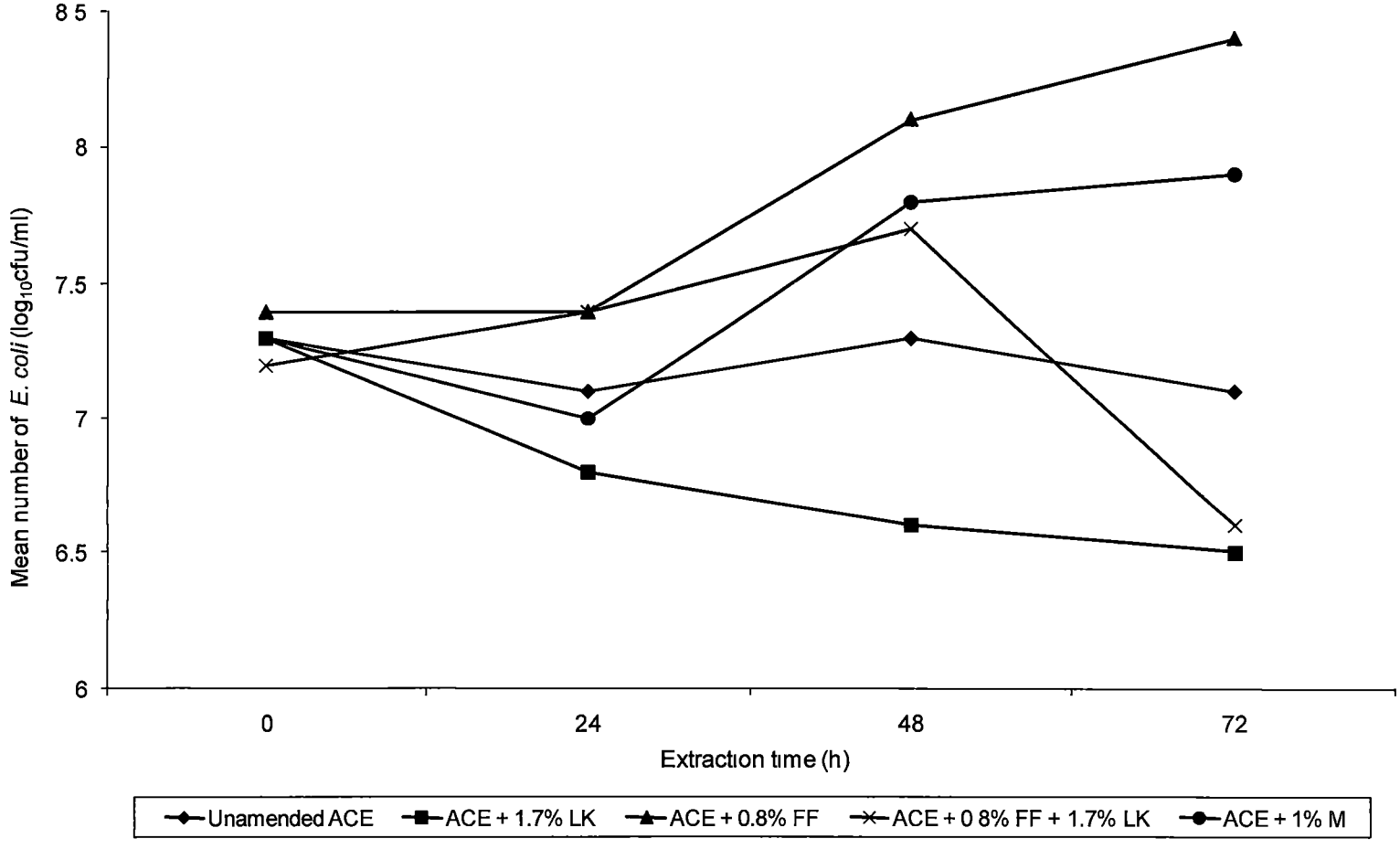


Figure 3.1 Experiment one: effect of ACE or ACE amended with molasses (M), liquid kelp (LK), fish hydrolysate (FF), or a mixture of fish hydrolysate and liquid kelp (FF + LK) on the persistence and multiplication of a non-pathogenic streptomycin resistant strain of *Escherichia coli* (log_{10}cfu/ml) up to 72 h after extraction commenced. At 0 h extraction time, each flask was inoculated with the *Escherichia coli* strain to a final concentration of ~1 x 10^7 cfu/ml. ACE was amended with nutrients 24 h after extraction. Significant differences among treatments were observed at 48 h extraction time (lsd = 0.21, $P < 0.001$) and 72 h extraction time (lsd = 0.50, $P < 0.001$).

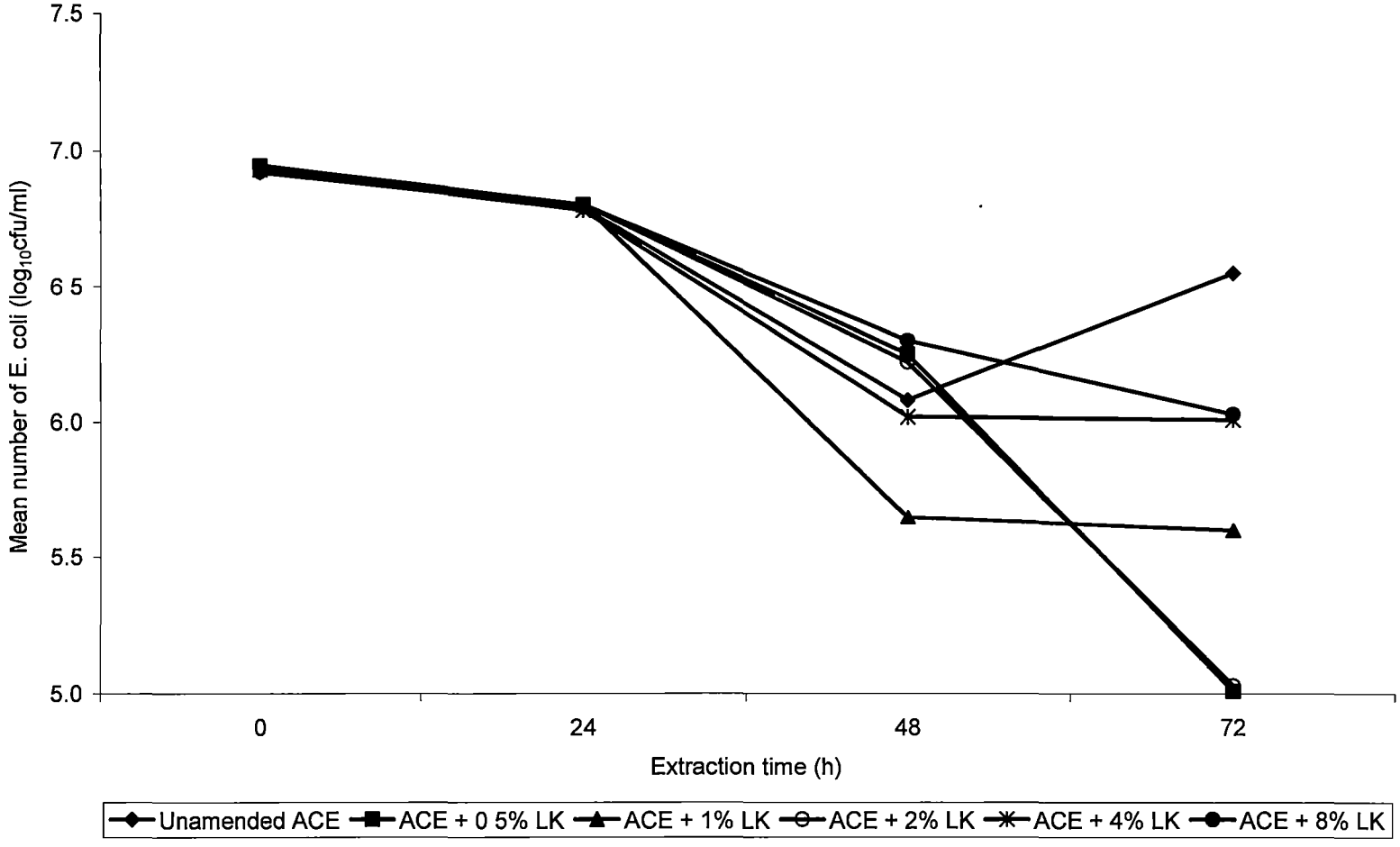


Figure 3.2 Experiment two: effect of ACE amended with various concentrations of liquid kelp (LK) on the persistence and multiplication of a non-pathogenic streptomycin resistant strain of *Escherichia. coli* (log_{10}cfu/ml) up to 72 h after extraction commenced. At 0 h extraction time, each flask was inoculated with the *Escherichia coli* strain to a final concentration of ~1 x 10^7 cfu/ml. ACE was amended with nutrients 24 h after extraction commenced. Significant differences among treatments was observed at 48 h extraction time (lsd = 0.36, $P = 0.021$) and 72 h extraction time (lsd = 0.24, $P < 0.001$).

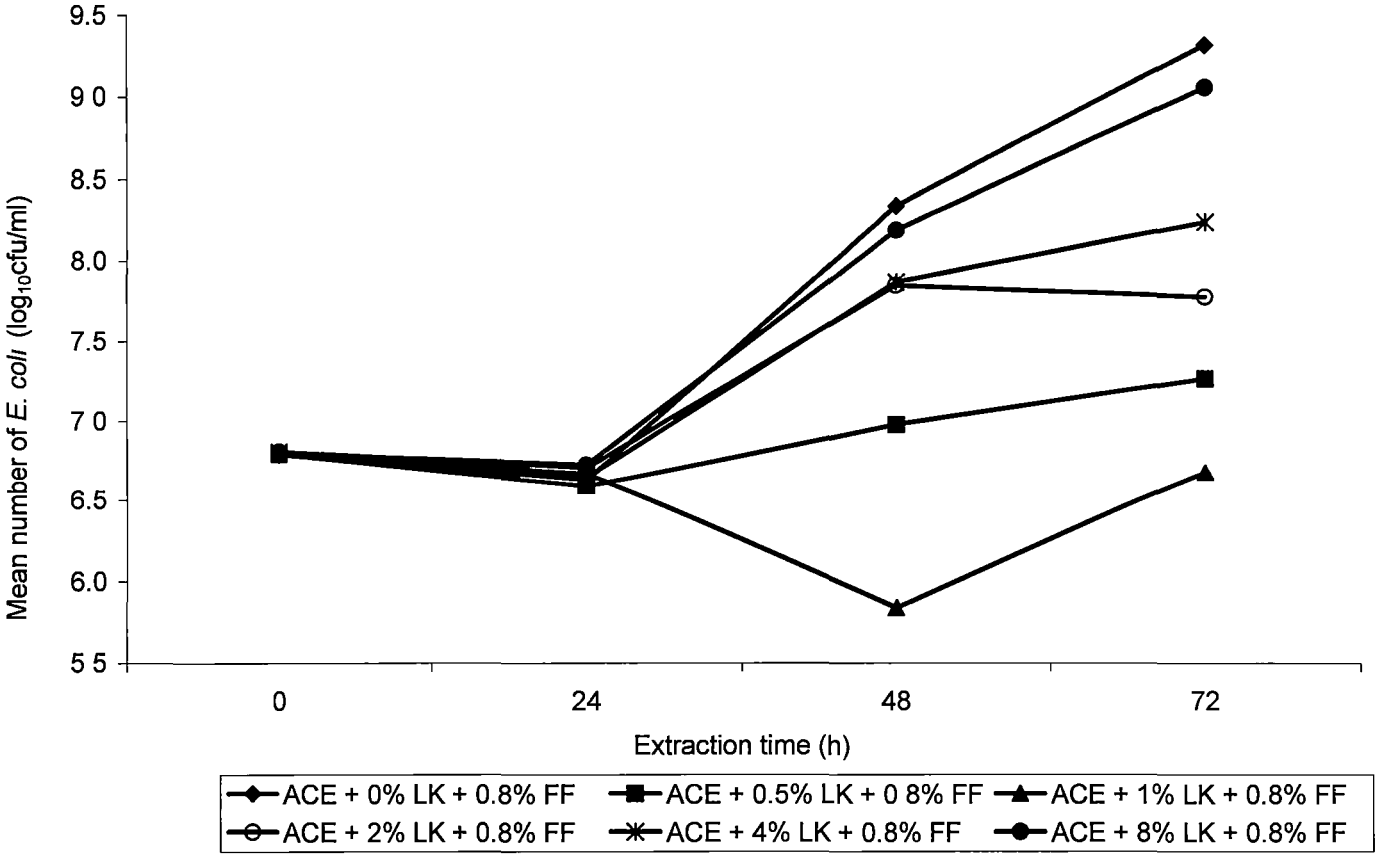


Figure 3.3 Experiment three: effect of ACE amended with 0.8% FF and various concentrations of liquid kelp (LK) on the persistence and multiplication of a non-pathogenic streptomycin resistant strain of *Escherichia. coli* (log_{10}cfu/ml) up to 72 h after extraction commenced. At 0 h extraction time, each flask was inoculated with the *Escherichia coli* strain to a final concentration of ~1 x 10^7 cfu/ml. ACE was amended with nutrients 24 h after extraction commenced. Significant difference among treatments was observed at 48 h extraction time (lsd = 0.15, P <0.001) and 72 h extraction time (lsd = 0.03, P <0.001).

As illustrated in Fig. 3.1, there appeared to be an increase in the number of streptomycin resistant *E. coli* after 48 and 72 h of extraction when 0.8% FF was added to ACE. Moreover, the highest mean number of *E. coli* in experiment three was observed when ACE was amended with 0.8% FF, without addition of LK (Fig. 3.1). The addition of 0.5% and 1% LK appeared to negate the effect of 0.8% FF (Figs 3.1, 3.3). However, where 0.8% FF was added to various concentrations of LK (Fig. 3.3), the mean number of *E. coli* generally increased with increasing concentration of LK, and the mean number approached that observed in the 'FF only' treatment when the concentration of LK in the nutrient mixture was 8%.

The number of culturable bacteria did not differ significantly across treatments in experiment three (Table 3.4). There appeared to be a small but statistically insignificant increase in the number of culturable fungi with increasing concentration of LK.

In all three ACE experiments, there was no significant change in the total number of culturable bacteria when there was a significant change in the number of pathogenic streptomycin resistant *E. coli.* Overall, the numbers of culturable bacteria or fungi in the various ACEs did not appear to be related to the number of *E. coli.*

3.5 Discussion

Supplementing standardised ACE with molasses or fish hydrolysate can lead to an increase in *E. coli* populations when compared to inoculated, unamended ACE. This result is consistent with the findings of other studies where the multiplication of enteric pathogens in amended ACE has been demonstrated (Duffy *et al.* 2004, Kannangara *et al.* 2006, Ingram and Millner 2007). In contrast to other research, this

was the first study to prepare ACE from compost in the early secondary mesophilic stage rather than the late mesophilic (mature) stage of aerobic composting. Furthermore, this study was the first to measure the number of culturable bacteria in every ACE treatment to determine if nutrient amendments altered total bacterial numbers or just the inoculated strain of *E. coli*. Kannangara *et al.* (2006) measured bacterial densities in ACE with and without carrot juice but not in ACE amended with kelp or molasses. Culturable fungi were also enumerated in every ACE treatment in this study and served to describe how nutrient amendments influenced fungal abundance.

ACE production methods and experimental systems used to enumerate human pathogenic bacteria have been variable across studies, making comparisons difficult. In both this study and the study of Kannangara *et al.* (2006), a high inoculum load was employed to ensure that growth of the challenge strain, if possible, would be able to be observed and not overwhelmed by other organisms already present in the test batch of ACE produced under conditions that simulated commercial practice. In contrast, the method used by Duffy *et al.* (2004) was limited because only one cfu of *E. coli* or *S. enterica* was added to the extract, limiting the chance for bacterial strain persistence and multiplication. In future, the significance of the *E. coli* inoculum concentration in this study could be assessed by application of a range of inoculum doses. Indeed, the compost industry requires a standardised method for determining the potential for human pathogen presence and growth in ACE and amended ACE.

There is a common perception that the natural microbial community in ACE amended with nutrients outcompetes human pathogenic bacteria (Ingham 2000). In both the studies conducted by Duffy *et al.* (2004) and Kannangara *et al.* (2006), there

was a positive correlation between the concentration of molasses and *E. coli* numbers and the speculative conclusion was that the natural microbial community did not prevent the growth of *E. coli*. The study reported here confirms this result given that there was a greater number of *E. coli* in ACE amended with molasses or fish hydrolysate than any other treatment (Table 3.4), while the number of culturable bacteria did not change when ACE was amended with these nutrients. Furthermore, there did not appear to be any relationship between the number of fungi across ACE treatments tested and the number of *E. coli*. Both ACE amended with liquid kelp (LK) or molasses (M) had a significantly greater number of fungi than unamended ACE at 72 h extraction time; however, ACE amended with M supported the growth of *E. coli* whereas ACE amended with 0.5% to 2% LK inhibited the growth of *E. coli*.

The abundance of culturable bacteria was not significantly correlated with changes in conductivity, pH, oxygen and nitrate levels across treatments and extraction times. The results in Chapter 1 illustrated that the bacterial diversity, rather than abundance, was most likely important for pathogen suppression by ACE and perhaps bacterial diversity changed in response to changing environmental conditions in this study. Analyses of bacterial diversity across treatments and extraction times would be necessary to test this hypothesis. There was a significantly lower quantity of dissolved oxygen, a lower pH and a greater conductivity in ACE amended with M or FF than unamended ACE (Table 3.2D). These conditions may have favoured growth of facultative anaerobes, including *E. coli,* as discussed by Kannangara *et al.* (2006), given that *E. coli* primarily inhabits the digestive system of animals, which has a low level of dissolved oxygen. Furthermore, *E. coli* and other bacteria in ACE may have metabolised the simple carbohydrates present in molasses and amino acids and

peptides present in FF consequently consuming oxygen and produced acidic end products.

The significant decline in *E. coli* populations from 0 h to 72 h extraction time when 0.5% to 2% LK was introduced to standardised ACE (Fig. 3.1) was in contrast to the results of Kannangara *et al.* (2006), where there was a positive correlation between *E. coli* numbers and the kelp supplement up to a concentration of 0.8%. Differences in the formulation of kelp (powdered or liquid) used in each study might explain the contrasting results. According to Whiting *et al.* (2007), powdered kelp is lower in plant growth-promoting hormones and macro- and micro-nutrients than cold processed liquid kelp, although how these differences in composition would have led to contrasting results with regard to *E. coli* growth remains obscure. Previous research on kelp extracts has shown antibacterial activity against a variety of pathogenic microorganisms, including gram-positive and gram-negative bacteria such as *Staphylococcus* sp., *Brucella* sp., *Bacillus* sp., *Klebsiella* sp., *E. coli* and *Salmonella* sp. (Mautner *et al.* 1953). Mautner *et al.* (1953) suggested that the antibiotic activity was due to the presence of phenolic compounds commonly found in kelp. Research by Yuan and Walsh (2006) verified this suggestion and revealed a correlation between antioxidant activities and amino and phenolic acids in kelp extracts.

According to Leibig's Law of the Minimum, the growth of an organism is controlled by the scarcest resource (van der Ploeg *et al.* 1999). Understanding how ACE amended with particular nutrients either promotes or inhibits *E. coli* growth, will further define conditions under which ACE can be prepared that reduces the risk of human pathogen contamination and multiplication. The inhibitory effect of LK on *E.*

coli populations diminished with increasing concentration of LK in ACE, especially by 72 h after extraction (Fig. 3.2A, B, C, D). Similarly, when the concentration of LK in ACE increased above 2% in the presence of 0.8% FF (Fig. 3.3), there appeared to be a threshold concentration of LK, above which the growth of *E. coli* was no longer reduced relative to ACE amended with 0.8% FF. These results, when taken together with the decline in fungal numbers when the LK concentration increased above 2%, suggest complex dynamics of individual microbial taxa as determined by availability of macro- and micro-nutrients, growth promoting substances and/or production of antibiotics.

In summary, *E. coli* multiplied *in vitro* when inoculated standardised ACE prepared from compost in the early mesophilic phase of composting was extracted for 72 h and amended with M or FF. Conversely, there was a decline in *E. coli* numbers when ACE was amended with up to 2% LK. The interaction between nutrients added to ACE could be examined further by including additional control treatments, such as the individual nutrients in the absence of ACE. Further analyses, such as microbial diversity in different ACEs or the presence of antibiotics in nutrient supplements, in particular LK, might aid interpretation of results. The reason for some changes in direction of trends of *E. coli* numbers between 48 h extraction time and 72 h extraction time also needs to be assessed and the potential for pathogen survival on the leaf and fruit surface following ACE application. Results of this study on standardised ACE, and earlier research on *E. coli* growth and persistence in ACE (Duffy *et al.* 2004, Kannangara *et al.* 2006, Ingram and Millner 2007), suggest the potential for enteric pathogens to multiply if amended ACE was contaminated; however, the probability that enteric pathogens in ACE survive the transfer to the surface of fruit and foliage and are ingested by humans at a harmful level remains

unknown. In the meantime, ACE should be prepared from compost made according to the Australian Standard and using occupational, health and safety practices. This will reduce the risk of enteric pathogens contaminating ACE and multiplying to levels that present a danger to human health.

GENERAL DISCUSSION

The results presented in this dissertation suggest that aerobic compost extract (ACE) has the potential to be incorporated into an integrated management strategy for sustainable control of two diverse grapevine pathogens: *Erysiphe necator* which causes powdery mildew and *Botrytis cinerea* the causal agent of botrytis bunch rot. This is the first study to standardise the production of ACE prior to field evaluation and to demonstrate disease control in wine grapes produced commercially in a variable cropping environment.

The first step of this research was to define qualities of compost and an extraction process that could produce aerobic compost extracts that consistently inhibited the growth and sporulation of *B. cinerea in vitro* (Chapter 1). A detached bean leaflet assay was utilised as a model experimental system to determine the qualities of compost and extraction parameters necessary for maximum pathogen suppression before evaluating ACE on whole plants in Chapter 2. The system was initially developed by Bouhassan *et al.* (2004) and modified for evaluating ACE and assessing plant symptoms by the use of image analysis. The assay separated ACE treatments efficiently and reproducibly for their impact on the growth and sporulation of *B. cinerea*, including the effect of varying compost to water ratios and the temperature of compost used to prepare ACE. This technique has the potential to be adopted across horticulture for testing ACE and for screening single-target biological control agents for *B. cinerea* and possibly other necrotrophic plant pathogens. This rapid bioassay also provides an alternative to other techniques used to evaluate ACE in relation to *B. cinerea*, for example, the inoculation of potted plants of geranium described by Scheuerell and Mahaffee (2006). A significant finding of this research was that the bean leaflet assay identified production

conditions for ACE which led to suppression of two grapevine diseases of diverse epidemiology and pathogen biology; namely, grapevine powdery mildew and botrytis bunch rot. In particular, *B. cinerea* was suppressed on bean leaflets under controlled conditionsand powdery mildew, caused by a different pathogen species (*E. necator*) with a different mode of nutrition, was suppressed on on grapevine leaves and berries in a variable field environment.

There are a large number of variables involved in the production of ACE (Scheuerell and Mahaffee 2002). The most suppressive and consistent extracts were generally produced from compost in the early secondary mesophilic phase of composting when internal windrow temperatures were between 40°C and 50°C. The compost ingredients did not appear to be important for determining the activity of ACE, rather a great diversity of microorganisms as illustrated by T-RFLP and/or the presence of biological control agents in ACE appeared to determine the level of pathogen suppression.

The major difference between the standardised ACE prepared in this research and the extracts reported in the peer-reviewed literature to date (Welke 2004, Scheuerell and Mahaffee 2004, 2006, Haggag and Saber 2007) is that ACE was prepared from compost prior to compost maturity. Aerobic windrow composting is a complex process whereby internal windrow temperature is the primary regulator of microbial diversity (Ishii *et al.* 2000, Peters *et al.* 2000, Anastasi *et al.* 2002, Tiquia 2005), activity (Ryckeboer *et al.* 2003a) and population structure (Hermann and Shann 1997). At temperatures between 40°C and 50°C in the early secondary mesophilic phase of composting thermophiles and mesophiles co-exist and, according to Ryckeboer *et al.* (2003b), the relative abundance (cfu) of bacteria, actinobacteria and

fungi is 30-40%, 30-35% and 20-25%, respectively. At this composting stage, the following conditions are observed: (1) the optimum temperature for thermophilic fungi that moderate levels of nitrogen (Finstein and Morris 1975), (2) a diverse range of bacteria that use numerous enzymes to degrade organic material and transfer soluble materials into bacterial cells (Ryckeboer *et al.* 2003b), and (3) a great diversity and abundance of actinobacteria (Fergus 1964, Finstein and Morris 1975, Amner *et al.*, 1988). In theory, ACE produced from immature compost with a diverse range of microorganisms might maintain effectiveness in a field environment when particular groups of microorganisms in ACE increase in frequency and environmental conditions become unfavourable for another group. This concept of microbial buffering capacity in relation to disease suppression in a variable field environment is similar to the proposal of Stewart (2001), who suggested application of multiple biological control agents to consistently suppress crop diseases.

To date, ACE practitioners (Touart 200, Litterick *et al.* 2004) and researchers (Welke 2004, Scheuerell and Mahaffee 2004, 2006, Haggag and Saber 2007) have focussed on adding nutrients to ACE with the intention of manipulating ACE to favour particular microorganisms. The *in vitro* germination results in Fig. 1.5 and Table 1.8 of Chapter 1 indicate that ACE amended with FF inhibits the germination of *B. cinerea* to a greater extent than unamended ACE. However, the field results in Chapter 2 show that standardised unamended ACE is just as effective as ACE amended with FF in limiting disease in the field. Furthermore, results of Chapter 3 illustrate that selected nutrients such as fish hydrolysate can enhance the growth of human pathogenic *E. coli.* Indeed, rather than producing an extract from mature compost that is manipulated to favour a particular group of biological control agents that suppress a particular plant pathogen, the standardised unamended ACE

developed in this thesis appears to have potential to limit a range of plant pathogens over a variety of crops.

The next step in the standardisation of ACE *in vitro* and *in vivo* will be to continue to investigate the diversity of microorganisms using T-RFLP across compost windrows with variable initial ingredients, across compost extraction times and various compost quantities in water. These analyses will provide information regarding the threshold level of microbial diversity necessary for consistent pathogen and disease suppression and might also indicate how much diversity can change and still provide the same level of suppression. The addition of primers for actinobacteria (Conn and Franco 2004), known to be present in early secondary mesophilic compost (Herrmann & Shann 1997), will also help to interpret microbial ecology in composts between 40°C and 50°C. Measurements of microbial biomass, by extracting organic carbon (Vance *et al.* 1987), and microbial activity, by FDA and β-glucosidase analysis (Bandick and Dick 1999; Ryckeboer *et al.* 2003a), should be completed in conjunction with T-RFLP analysis of ACE and the results correlated with aspects of the early secondary mesophilic compost and pathogen suppression. In this thesis, nitrate was often absent from ACE prepared during the early secondary mesophilic stage of composting with nitrification increasing as composting proceeded to maturity. To ensure that the composting process is consistent and on target biologically and chemically, daily measurements of microbial activity by carbon dioxide analysis, plus temperature and moisture levels in the compost windrows will be necessary, as well as weekly measurements of nitrate, nitrite and ammonium, hydrogen sulphide, conductivity and pH.

This research has highlighted the need to introduce new terminology; namely, "broad-spectrum" ACE and "prescriptive" ACE. Broad-spectrum ACE refers to unamended ACE prepared from immature compost, as described in this research, and prescriptive ACE refers to ACE modified to increase the frequency of particular microbial taxa for targeting a specific pathosystem. Both broad-spectrum and prescriptive ACE might be integrated into disease management, with prescriptive ACE targeting a key stage in the life cycle of a pathogen on a particular host. The next step in research might be a series of experiments to investigate ACEs amended with particular nutrients that favour certain actinobacteria or fungi that have been proven to have antifungal properties. The aim would be to produce extracts where all the nutrients present in the solution are converted to biocontrol agent biomass and end-products of metabolism, including antimicrobials. In this case, when ACE is applied to the crop canopy there will be no nutrients available for the plant pathogen to utilise. The duration of extraction will be crucial because the aim will be to apply ACE once the microorganisms have completed the exponential growth phase and utilised all monosaccharide sugars. If the primary mechanism of inhibition of prescriptive ACE is due to antimicrobial activity, in particular, secondary metabolites, then extending the ACE production time further into the stationary phase of microbial growth may be advantageous. On the other hand, if the primary mechanism of action is due to end-products of catabolism, i.e. organic acids, then the end of the exponential phase of growth will be the optimal ACE production time before other organisms metabolise the end-products (Brock and Madigan 1991). It is hypothesised that myriad mechanisms of action might be associated with broad-spectrum ACE, with the relative contribution of each mechanism varying depending on environmental conditions. In contrast, prescriptive ACE might be linked to fewer

mechanisms of action. Understanding the key mechanisms of action of prescriptive and broad-spectrum ACEs, will improve the way ACE is produced and applied.

Chapter 3 demonstrates the significance of ensuring product safety if prescriptive ACEs are developed. The results show that if more than one nutrient is added to ACE then the interactions become complicated. To fully understand the effect of nutrients added to ACE and interactions between nutrients, additional control treatments should be tested. Appropriate control treatments for analysing the growth of *E. coli*, or other microbial taxa such as yeasts and actinobacteria, should include various concentrations of each nutrient supplement in aqueous solution, in the absence of ACE, and for composite nutrient supplements, the individual chemical components of the supplement.

The next phase of ACE research will require further testing of the effectiveness of ACE and developing ACE for application in commercial horticulture. A perceived or real risk associated with relying solely on multiple applications of ACE across a growing season could be addressed by including ACE in an integrated disease management strategy. Synthetic fungicides or single biocontrol agents such as *Trichoderma* sp. (Elad 1994) or *Ulocladium* sp. (Reglinski *et al.* 2005) could be applied during flowering to prevent *B. cinerea* infection in the grape florets, while ACE could be applied early and late in the season for powdery mildew control on the leaves. Compatibility of ACE with existing crop protectants needs to be investigated concurrently.

While microorganisms in ACE are necessary for disease control, particular microorganisms in ACE may impact negatively for crop production and post-harvest

processing. In wine making, fermentation can be inhibited by ingress of so-called 'wild' or foreign yeasts (Pandey 2004). The potential of ACE as a source of deleterious yeasts on grape berry surfaces needs to be investigated prior to application to commercial vineyards. PCR-RFLP of the ITS region of 5.8 rRNA genes and/or selective growth media for various yeast genera could be used to characterise yeast microflora on grape berries after application of ACE (for example, refer to Combina *et al.* 2005). Understanding the persistence of microbial populations from ACE in the frutosphere will determine the minimum interval between the last application of ACE in the vineyard and the harvest date that prevents potential, but as yet unquantified, negative impacts of ACE on wine making.

It is hoped that communication of the results obtained in this thesis will raise awareness among compost producers about the importance of quality control during composting for production of ACE that suppresses disease consistently. ACE offers a potential extra market for compost, which might enable existing and prospective compost producers to realise profitable business plans. Extra business can also be generated through consultancy to horticultural producers on how to integrate ACE into disease management. Additional field experiments investigating ACE across horticultural crops, regions and seasons, using commercial practices and equipment, will provide practical and concrete evidence to industry on the benefits of ACE. Researchers and practitioners will need to develop practical strategies for integrating ACE with current technologies. Adoption of ACE will depend on the cost and availability of waste products for composting as well as consultants and end-users being receptive to the idea of biological control and the adoption of sustainable practices in general. Key drivers that motivate change from conventional disease

management and mechanisms for change will need to be communicated and implemented. Therefore, the uptake of ACE will depend not only on demonstrated effectiveness, but also on well-targeted extension so that the technology does not remain on the 'academic shelf'.

Commercialisation of standardised unamended ACE for crop protection may be a difficult task considering the registration process for fungicides. This process requires well-defined active ingredients, with known human toxicology and predictable mechanisms of action. Standardised ACE is a heterogeneous product of unknown chemistry, and contains a variety of live microorganisms whose diversity is likely to vary from batch to batch. Myriad mechanisms of action are likely, with specific mechanisms likely to dominate under certain environmental conditions. However, if retail markets for horticultural produce increase their demand for products free of fungicide residues then there may be justification for developing new criteria and regulations for approving the use multiple-target biological controls such as standardised ACE. The final paragraph of this thesis sets out some preliminary criteria for managing risk in relation to standardised ACE production and application. These recommendations assume that the effectiveness of ACE for managing a specific plant disease has been demonstrated over multiple sites and seasons.

Preliminary Recommendations

1. Production of compost according to the Australian Standard with a C:N ratio at compost windrow initiation of 25-30:1 and daily and weekly testing of compost quality.

2. Production of ACE using compost from the early secondary mesophilic stage of (aerobic) composting and compost to water ratios between 1:3 and 1:30.

3. Maintenance of batch to batch quality control by bioassay for pathogen suppression.

4. Specification of a 'use-by' date for ACE and certification that the batch tested negatively to indicator species of human pathogens or at a level below some threshold concentration considered to pose a threat to workers or final product safety.

5. After further research, specification of crop-specific spray intervals, methods of integrated disease management and horticultural equipment suitable for the application of ACE. In particular, equipment including spray tanks should be cleaned appropriatelyto avoid undesirable microbial growth and blockage of spray nozzles or other narrow orifices.

6. Specification of crop-specific withholding periods (the time between the last application of ACE and harvest) or crop phenological stages when ACE application should be avoided, if further research indicates any negative impacts on fresh or processed crop quality. In the case of wine grapes, the effect of ACE application on wine quality should be assessed.

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