Biological control of eutypa dieback of grapevines: interactions between the pathogen and fungal antagonists

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ABSTRACT

Biological control of eutypa dieback of grapevines using *Trichoderma harzianum* was investigated in laboratory, glasshouse and field experiments. *Fusarium lateritium*, a fungus known to be an effective antagonist of *E. lata*, was also used in some experiments.

T. harzianum inhibited mycelial growth and germination of ascospores of *E. lata* by antibiosis on potato dextrose agar medium. The three strains investigated inhibited mycelial growth by production of both volatile and non-volatile antibiotics, although the degree of inhibition varied between strains of the antagonist and between isolates of the pathogen. The non-volatile antibiotics had a fungistatic effect on some isolates of *E. lata* and a fungicidal effect on others. Scanning electron microscopic examination of co-inoculated gamma-irradiated grapevine cane segments and of co-inoculated 1-year-old-canes placed in water-saturated rockwool in the laboratory showed hyphae with loss of turgor and collapse, abnormal swelling, winding and parallel growth.

Significant reduction in infection by *E. lata* was detected *in vitro* when autoclaved or gamma-irradiated canes were inoculated with mycelial plugs or spores of both pathogen and antagonist. When pruning wounds on 1-year-old canes of cultivar Shiraz in the glasshouse were treated with spores of *T. harzianum*, then challenged 2 and 7 days later with mycelial plugs of *E. lata*, infection by *E. lata* was reduced significantly at both times. The pathogen was recovered from 13-38% and 0-25% of the canes treated with the antagonist and challenged 2 and 7 days later, respectively, with the pathogen. *E. lata*

was recovered from all of the controls at both times of treatment. Furthermore, in 12 weeks *T. harzianum* colonised the canes up to 10 cm below the point of inoculation.

In the vineyard, five trials were established to test pruning wound treatments over 3 years using the cultivars Cabernet Sauvignon, Shiraz, Rondella and Palomino. Treatment of pruning wounds with *T. harzianum* or *F. lateritium* protected vines from infection by ascospores of *E. lata*, in most experiments, when the wounds were challenged 24 hours or 14 days after treatment with the antagonist. In most of the pruning wound trials, the application of spore suspensions of the antagonists significantly ($P<0.001$) reduced infection by the pathogen. The percentage recovery of *E. lata* from the control vines that had been inoculated with *E. lata* alone was low in four of the five trials. Also, colonisation of vines by *T. harzianum* was studied following insertion of *T. harzianum*-impregnated wooden dowels (Trichodowels®) into holes drilled 30 cm above ground level into trunks of vines of cultivar Nyora. *T. harzianum* was re-isolated from four of the seven vines 20 months after inoculation and had grown 18 cm above the point of inoculation in two of the vines during this time.

Results suggested that *T. harzianum* has potential in the control of eutypa dieback of grapevines when used as a pruning wound treatment. While results of experiments with the Trichodowels® were encouraging, there is a need for more detailed studies of their efficacy in preventing infection of vines by *E. lata*.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis being made available for loan and photocopying when deposited in the University Library.

Signed **Date** 19/11/03

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CHAPTER 1. INTRODUCTION

Eutypa dieback is a destructive canker disease caused by the ascomycetous fungus *Eutypa lata* (Pers.: Fr.) Tul. & C. Tul. (syn. *Eutypa armeniacae* Hansf. & M. V. Carter) (Moller and Kasimatis, 1978; Moller and Kasimatis, 1981a). This disease affects woody plants in 88 species within 28 families and is widely distributed in the temperate regions around the globe (Bolay and Carter, 1985; Carter, 1991). Grapevine (*Vitis* spp.), apricot (*Prunus armeniaca* L.), almond (*P. dulcis* (Mill.) Webb), apple (*Malus domestica* Borkh.) and sweet cherry (*Prunus cerasus* L.) are some of the hosts of agricultural importance.

This disease is most prevalent in older vines and in areas that receive an annual rainfall of more than 500 mm (Wicks and Hall, 1997). The disease is usually rare until a vineyard reaches the age of 10-12 years after which the incidence increases until almost all the vines are infected at 20 years (Duthie *et al.*, 1991; Munkvold and Marois, 1995). Eventually the disease kills the vines and, therefore, reduces the longevity of the vineyards.

Significant yield losses are caused by eutypa dieback in grape-growing areas throughout the world (Carter, 1991). Wicks and Hall (1997) have suggested that in Australia up to 60 % of the vines may be affected in some old, elite vineyards. Estimates made in North America show yield losses of 30-62% (Munkvold *et al.*, 1994). In South Australia the disease is common in Barrosa Valley, Coonawarra, Clare Valley, Eden Valley and McLaren Vale areas. Wicks and Davies (1999) estimated yield loss of more

than $2,800 per hectare in Shiraz, in Eden Valley, where 47% of the vines showed symptoms of infection.

Treating wounds with fungicides or wound sealants is the preferred method of control, since the pathogen enters its host through pruning wounds (Moller and Kasimatis, 1978; Moller and Kasimatis, 1981b). Eradicative sanitation methods are not always successful because of the broad host spectrum of the pathogen (Carter, 1991). Benomyl has been reported to be effective against *E. lata* (Munkvold and Marois, 1993b), but this fungicide was never registered in Australia as a grapevine wound protectant. The manufacturers withdrew benomyl from the market in 2002. Also, pruning wounds may remain susceptible for 4 weeks (Munkvold and Marois, 1994), hence chemical treatments may not protect the wound for the entire period of susceptibility. Biological control agents capable of colonising pruning wounds and the tissues below the wounds may provide better long-term protection than fungicides.

Biological control of *E. lata* on grapevines has been demonstrated using *F. lateritium*, *Cladosporium herbarum* and *Bacillus subtilis* (Ferreira *et al.*, 1991; Munkvold and Marois, 1993a), however, none of these have yet been developed into commercial products. Trichoprotection® products, manufactured by Agrimm Technologies Ltd, New Zealand, containing seven strains of *Trichoderma harzianum*, have been registered for protection against the silver leaf pathogen *Chondrostereum purpureum* on stone fruit trees since 1991, and have been suggested to be effective against *E. lata* on grapevines (Hunt, 1999). The potential for biological control of *E. lata* using *T. harzianum* was investigated in this project.

The aims of this study were to: (1) elucidate mechanism/s of inhibition of *E. lata* by *T. harzianum in vitro*; (2) investigate interactions of pathogen and antagonist on grapevine tissues in the laboratory; (3) study inhibition of *E. lata* by *T. harzianum* in grapevine cuttings in the glasshouse; (4) investigate the potential of *T. harzianum* as a biological agent to prevent infection by *E. lata* in the vineyard, with emphasis on pruning wound treatments and (5) study the wound response of the host to understand interactions between host, pathogen and antagonist.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

Eutypa dieback may be managed by treating pruning wounds with fungicides and delaying pruning till late in the dormant season (Moller and Kasimatis, 1980; Moller and Kasimatis, 1981b; Petzoldt *et al.*, 1981). These management practices, however, are only partially effective. Sanitation pruning is usually used in premium wine-growing areas, but is labour intensive and expensive. In view of the threat of eutypa dieback to the sustainability of South Australian vineyards and the possibility of the development of fungicide resistant strains of the pathogen, there is an urgent need to develop a suite of effective control measures including cultural, chemical and biological control methods.

The literature on eutypa dieback and related trunk diseases of grapevines is reviewed in this chapter by dividing the relevant information into four main categories. The first section reviews the literature on the pathogen in terms of its historical background, taxonomy and nomenclature, biology, genetic variability, symptoms of the disease and the environmental factors that affect disease development. The second section deals with the host. In the third section the various control practices are discussed with emphasis on research using *Trichoderma* spp. in biological control. Other trunk diseases of grapevines are reviewed in the fourth section.

2.2. The pathogen

2.2.1. Historical background

Eutypa dieback was initially recognized in the 1920s in Australia as a disease of apricot trees. In the South Australian Journal of Agriculture in 1924 and 1925, it was reported that old apricots in many parts of the southern district were losing big limbs and this was attributed to "root trouble" (Carter, 1991). Dowson (1931) provided the first detailed description of the disease, stating that it was a definite dying back of both young and old branches, with fungal mycelium invading the dead or dying tissue. The presence of this fungus was thought to cause the trees to produce gum to such an extent that the sap conducting vessels of the wood became clogged and the water supply was affected, resulting in the death of branches. The infection could be traced to old pruning wounds (Dowson, 1931). Harris (1932) observed the same symptoms in the Barossa district of South Australia, and called the condition “gummosis”. Adam (1938) confirmed the tentative diagnosis of Samuel (1933) of the conidial stage of the pathogen as *Cytosporina* sp. However, the mode of reproduction and dissemination remained obscure until 1956, when the sexual stage, *Eutypa armeniacae* Hansf. & Carter, was identified at the Waite Agricultural Research Institute in South Australia (Carter, 1957a).

Eutypa armeniacae was first reported as a saprophyte on grapevine in 1957 in Adelaide, South Australia (Carter, 1957b). A decade later, Moller *et al.* (1968) found it on dead arms of grape in California. Meanwhile, in Europe, the term “apoplexy” was widely used to describe any disease manifest by sudden wilting of leaves of apricot trees, which was found to be widely distributed and increasing in frequency. No single

organism had been consistently associated with the disease and, therefore, it was attributed to numerous and diverse physiological and pathological factors (Carter, 1991).

Reports of dieback symptoms in apricot trees in the 1970s in Spain, Italy, Greece, Bulgaria and Libya confirmed the presence of *E. armeniacae* in Europe and the Mediterranean region but not a single record of the pathogen existed in the UK until 1985 (Carter, 1991). Meanwhile, Carter and Price (1974) confirmed the pathogen in apricot trees in South Africa.

Eutypa dieback of grapevines was confused with other fungal disorders because of similarities of symptoms that had long been attributed to other pathogens such as *Phomopsis viticola* Sacc.. Research by Moller and Kasimatis (1978) revealed that *E. armeniacae* was the pathogen causing dieback symptoms on grapevine. The pathogen is now commonly known as *E. lata* (Carter, 1991).

2.2.2. Taxonomy and nomenclature of the pathogen

E. lata belongs to the Ascomycota and is classified in the single family Diatrypaceae of the order Diatrypales. The fungi grouped in this family produce asci with thin walls, long stalks and small, iodine-positive apical rings, in which pale brown or yellow allantoid ascospores are formed. Ascospores are 6.2 – 11 × 1.5 – 2 µm in size and aseptate. The asci are contained in perithecia, which usually have furrowed ostioles (Carter, 1991). The perithecia are produced in stromata on dead host tissue.

The teleomorph of *E. lata* develops in the wood as extensive stromata. The ostioles emerge separately, projecting 50 – 150 µm, are rounded or conical, never cruciform, and 120 – 180 µm in diameter. The single layered ascomata are closely spaced or in contact,

sometimes compressed together. These are spherical or ovoid in shape (300 -) 400 – 600 (- 700) μm in diam. with short necks. The anamorph is found as conidiomata on natural substrates. Conidia are produced in white to cream translucent masses on agar, single celled, filamentous, curved towards the obtuse apex and straight towards the truncate base, (14-) 20-35 (-40) μm long ×1- 1.3 μm thick whereas on natural substrates, conidia are (20-) 30- 45 (-50) μm in length (Carter, 1991). The anamorph of *E. lata* is now placed in the genus *Libertella* (Carter, 1991). The ascospores are responsible for the dissemination of the pathogen and conidia may act only as spermatia (Carter, 1991). However, some researchers observed conidial germination and have suggested that these can propagate disease (Belarbi and Mur, 1983; Ju *et al.*, 1991).

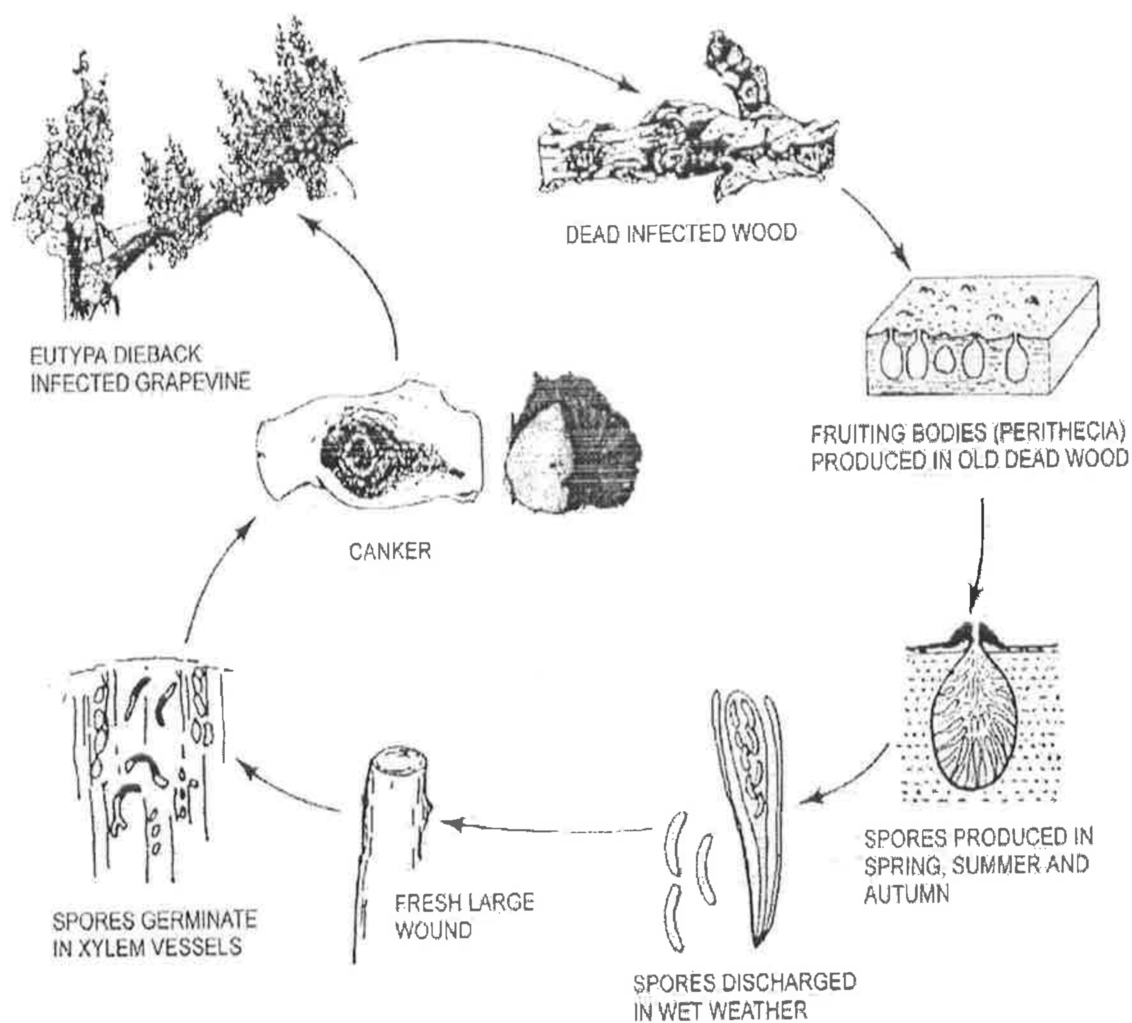
E. lata is one of the five species contained in the genus *Eutypa*, which was first established by Tulasne and Tulasne (Carter, 1991). Glawe and Rogers (1982) described the anamorphs of six members of the family Diatrypaceae in detail. *E. lata* and *E. armeniacae* were retained as separate species but it was noted that these two species differed very little or not at all in their morphological characteristics (Glawe and Rogers, 1982; Carter, 1991). More recently, De Scenzo *et al.* (1999) used molecular evidence to suggest that these are two different pathogenic species. Both species were capable of infecting native and cultivated hosts in California.

2.2.3. Biology of *Eutypa lata*

2.2.3.1. Disease cycle

Development of eutypa dieback (Figure 2.1) begins in grapevine when the ascospores enter the plant via exposed xylem vessels of fresh pruning cuts (Carter, 1960; Carter, 1965; Moller and Kasimatis, 1978). The ascospores germinate in the vascular tissues, and hyphae proliferate slowly within the vessels and later colonise the associated elements of the functional wood. The progress of the disease in grape is slow and no symptoms are seen for one or two seasons, after which foliar symptoms are manifested (Moller and Kasimatis, 1978). A canker is usually apparent by the third or fourth season after infection and many more growing seasons may elapse as the disease progresses and eventually kills the affected arm or cordon (Pearson and Goheen, 1988). Once death occurs, it takes several more years before perithecia are formed on the dead infected wood (Flaherty *et al.*, 1992). Ascospores are generally released during and soon after rainfall and are windborne to infect fresh wounds, in spring, summer and autumn. Rain is necessary for the release of the ascospores from the perithecia and their entry into the exposed ends of the xylem vessels (Pearson and Goheen, 1980). According to Carter (1957a), a minimum rainfall of 2 mm is necessary to initiate the liberation of ascospores when the stromata are dry. Wind is necessary for the aerial transport of ascospores. Studies by Pearson and Goheen (1988) suggested that ascospores are able to travel 50-100 km. Generally, dispersal occurs during overcast weather when solar radiation is minimal (Ramos *et al.*, 1975b).

Figure 2.1. Disease cycle of eutypa dieback (Flaherty *et al.*, 1992).



2.2.3.2. Histopathology

Infection is initiated when the ascospores are washed into fresh wounds on grapevine wood more than a year old. Ramos *et al.* (1975a) demonstrated that a single ascospore is sufficient to initiate infection. According to Moller & Kasimatis (1980), wounds that are made on 1 year-old wood are less susceptible to infection than those made on older wood. Generally, viable spores germinate within 12-24 h at the optimal temperature of 20-25°C, usually 2 mm or more beneath the wound surface (Carter, 1991). Symptoms develop 2 to 3 years after infection, when the fungus has colonised the vascular tissue of the arm and trunk of the vine (Moller and Kasimatis, 1981a; Duthie *et al.*, 1991). The pathogen releases phytotoxic compounds, one of which has been isolated and identified as eutypine, 4-hydroxy-3-(3-methyl-3-butene-1-ynyl) benzaldehyde (Tey-Rulh *et al.*, 1991). The toxin was believed to be responsible for the expression of disease symptoms (Mauro *et al.*, 1988; Tey-Rulh *et al.*, 1991). However, a recent report suggests that the fungus produces a suite of potentially toxic secondary metabolites that may cause the symptoms (Molyneux *et al.*, 2002).

2.2.4. Symptoms

Symptoms are seldom observed in vines younger than 6 years of age (Moller and Kasimatis, 1980). The disease is obvious in early spring when the new season's shoots appear stunted, deformed and discoloured.

Generally, the young leaves are small, chlorotic, often cupped with tattered margins (Moller and Kasimatis, 1981a). Brown speckles develop on leaves and the margins appear scorched (Pearson and Goheen, 1988; Flaherty *et al.*, 1992; Emmett and

Magarey, 1994). The shoots usually have "zig-zagged" internodes. The disease first appears on one or two spurs, but becomes more extensive with each passing year, killing the infected arm and eventually the entire grapevine. Formation of pruning wound cankers is an important diagnostic feature of dieback disease (Moller and Kasimatis, 1981a; Duthie *et al.*, 1991). The dead tissue surrounds old pruning wounds and appears as a wedge-shaped necrotic zone in the cross-section of the wood (Flaherty *et al.*, 1992). Creaser and Wicks (2002) reported that in severely affected vines the wood symptoms might progress into the trunks below ground level but do not extend into the root system. Flower clusters tend to shrivel and die on severely affected shoots. Clusters on shoots that are affected by the disease may have a mixture of large and small berries. Usually, symptoms are detected only after the fungus has become well established in the grapevine tissue. This makes disease management difficult.

2.2.5. Variability of the pathogen

E. lata exists as a range of genotypes that differ in virulence to individual hosts. This is expected since the pathogen is an ascomycete which relies exclusively on ascospores for its propagation and dissemination (Carter, 1991). Péros and Berger (1994) showed that isolates differ in their ability to causes symptoms. They demonstrated that some isolates that colonised grapevine cuttings did not induce foliar symptoms. Péros *et al.* (1997) also reported that *E. lata* isolates show a large variation in pathogenicity on cuttings in the greenhouse, but qualitative differences in pathogenicity of isolates did not correspond to the presence of symptoms in the vineyard. Differences in pathogenicity have been noticed among *E. lata* isolates from the same stroma/perithecium (Carter *et*

al., 1985; Rumbos, 1987; Péros and Berger, 1994) and even among single spore-derived isolates of the same ascus (English *et al*., 1983). Research has also revealed differences in pathogenicity between isolates from different geographic locations (Ramos *et al*., 1975a; Péros and Berger, 1994). Furthermore, genetic diversity in *E. lata* has been demonstrated recently using random amplified polymorphic DNA (RAPD) markers (Péros and Berger, 1999; Péros *et al*., 1999). Variation has also been observed in cultural traits (Glawe *et al*., 1982; English *et al*., 1983; Rumbos, 1987; Peros and Berger, 1994). This diversity has to be taken into account when devising disease control strategies. Differences in virulence of *E. lata* have been demonstrated on apricots (Ramos *et al*., 1975a; Carter *et al*., 1985).

Carter (1991) has also mentioned the possibility of hypovirulence transmission between isolates, which might render virulent strains ineffective when they are co-inoculated with an avirulent strain. He considered that hypovirulence could be a dominant character in *E. lata*. Apart from Carter's observations, not much is known about interactions between the different pathotypes of *E. lata*. This aspect needs further investigation, as hypovirulence could be useful in devising effective management strategies.

2.2.6. Environmental factors that affect disease development

It has been established that a two-fold process is involved in the release, dissemination and deposition of the propagules of the pathogen (Carter, 1965; Moller and Carter, 1965). Ascospore release occurs after rainfall and spores are disseminated and deposited by wind. Subsequent showers of rain aid in the redistribution of the

inoculum. The viable ascospores that reach the infection courts germinate, penetrate the vascular system and grow into woody tissue, where they are protected from the ever-changing environmental conditions. The major environmental factors that affect infection are temperature, relative humidity and rainfall.

2.2.6.1. Temperature and relative humidity

The time required for germination of ascospores is directly related to temperature. Carter (1991) showed that germination of freshly discharged ascospores was maximal at 20-25°C on agar within 11-12 h, whereas spores required more than 100 h to germinate at 2°C. Munkvold and Marois (1995) reported that the optimum conditions for germination and growth of *E. lata* are 22-25°C and at least 90% relative humidity (RH). According to Carter (1957a), if temperatures are below 15°C, the minimum RH must be maintained for longer periods of time for germination to occur. Apricot trees have been shown to become resistant to infection by *E. lata* when the mean maximum daily temperature was 20°C and RH 60 % ± 8 but were susceptible at the dormant temperature of 3°C (Ramos *et al.*, 1975a). Similar observations have been made in California, where infection by *E. lata* was less on grapevines pruned towards the end of winter, than on those pruned in early winter (Tresse *et al.*, 1982). Tresse *et al.* (1980) also demonstrated that *E. lata* infects pruning wounds on grapevines under controlled conditions at –1°C to +1°C, although infection under these conditions was infrequent. Their freeze and thaw tests also suggested that ascospores can germinate during winter and early spring, where temperatures alternate between above freezing and below freezing (e.g. -20°C) temperatures.

2.2.6.2. Rainfall

Liberation of ascospores begins within 3 h of rainfall, continues during the rainy period, and ceases after rainfall ends and stromata become dry (Pearson, 1980). Ramos *et al.* (1975b) demonstrated that a minimum rainfall of 1.27 mm was necessary for ascospore discharge. Tresse *et al.* (1980) reported the presence of octads of airborne ascospores after the stromata were exposed to a minimum of approximately 2 mm of rainfall at temperatures above 0°C. They also reported that free water (vine trunk wetness) maintained by prolonged rain resulted in continued ascospore dispersal for 24 h. The numbers of ascospore octads were high in the spring and declined in summer. In sub-freezing weather conditions during winter, when there was no rainfall, no ascospores were trapped (Tresse *et al.*, 1980).

Usually, eutypa dieback occurs abundantly in areas of mean annual rainfall of more than 600 mm, but is unlikely to develop where rainfall is less than 250 mm (Pearson and Goheen, 1988). Perithecia are exceedingly rare and the incidence of the disease is low in regions where the mean annual rainfall is below 279 mm (Carter, 1957a). Similar observations of the relationship between mean annual rainfall and the distribution and occurrence of the perithecial stage have been made by Ramos *et al.* (1975b). Pearson (1980) suggested that ascospore release may also be triggered by natural snowmelts. Ramos *et al.* (1975b) demonstrated that wind direction and speed also have an effect on numbers of airborne ascospores. Many ascospores were observed in the atmosphere when strong winds (8 - 11 km/h) followed rain, but light wind conditions (2 km/h) following rain did not favour the aerial transport of ascospores (Ramos *et al.*, 1975b).

Rainfall and wind speeds are, therefore, important factors that need to be taken into account when pruning, to minimise the risk of infection.

2.3. The host plant

Grapes belong to the family Vitaceae, genus *Vitis*. The Eurasian species, *Vitis vinifera*, comprises the majority of genetic material (apart from rootstocks) used for viticulture in Australia and in many other grape-growing countries (Dry and Gregory 1988; Pearson and Goheen, 1988). *V. vinifera* has become very diverse with time and about 5,000 cultivars exist today. Of these, only a small proportion are used commercially. Cabernet Sauvignon, Chardonnay, Chenin Blanc, Grenache, Merlot, Riesling, Shiraz and Sultana are some of the grape varieties in commercial use in Australia.

Cultivars vary in their susceptibility to the disease (Mauro *et al.*, 1988; Carter, 1991; Tey-Rulh *et al.*, 1991). However, Chapius *et al.* (1998) reported that there was no difference in susceptibility to infection by *E. lata* between cultivars, although under field conditions they differ in symptom expression. Further investigations are necessary to establish whether these differences in susceptibility are due to physiological or other factors. Also, the role of plant cell defence reactions in preventing or limiting invasion by *E. lata* in the tolerant cultivars is not yet known. Furthermore, information is lacking on the effect that the toxins produced by the pathogen have on the various cultivars of grapevine.

2.3.1. Yield loss

E. lata causes severe yield losses in grapevines around the globe. Yield loss estimates varied from 62 to 94% in severely affected vines on North America, while in moderately affected vines losses ranged from 19 to 50% (Johnsen and Lunden, 1985; Munkvold and Marois, 1994; Irelan *et al.*, 1999). According to Irelan *et al.* (1999), a poll in the California grape growing regions revealed that, of all the vineyard disease management practices during the period 1996-99, eutypa disease control was the most expensive, with the disease prevention costs being 72% greater than that for powdery mildew, the next closest ranking disease. French research indicates that within the 44,000 cognac-producing stocks examined in the Charentes vineyard, 20% of the mature grapevines were affected by the dieback disease in 1988. In the Bordeaux area, loss of 80 - 90 % has been reported in some vineyards (Dubos, 1987a).

In Australia, yield losses have been estimated to range from 0.6 kg to 9 kg per vine depending on the severity of the disease and losses may be in excess of $ 2,800 per hectare, as reported for Shiraz in the Eden Valley (Wicks and Davies, 1999, see Chapter 1).

2.4. Management of eutypa dieback

2.4.1. Cultural practices

Research indicates that special attention has to be paid to the timing of pruning. Pruning vines in late winter when ascospore production is low reduces the risk of infection (Ramos *et al.*, 1975b). Petzoldt *et al.* (1981) found that vines pruned towards

the end of the dormant season are less susceptible to infection by *E. lata* and that susceptibility of the wounds declines more rapidly in early spring compared to early winter. It has also been recommended that, where possible, top working of vines should be done in dry weather. Research is currently taking place in California to evaluate the influence of different canopy training systems in the management of the disease (Burnham, 1998; Lake *et al.*, 1998).

Other recommendations for management of eutypa dieback include detection and removal of diseased arms in early spring before healthy shoots obscure diseased shoots, removal and burning of affected plant debris and removal of diseased stumps that are above soil level. Remedial surgery of diseased arms is carried out by making a series of saw cuts until there is no evidence of stained wood. This will have a better chance of success if it is carried out before the disease has progressed extensively through the grapevine (Anon., 1997; Flaherty *et al.*, 1992; Emmett and Magarey, 1994). Reconstructing vines is yet another management practice. There are different ways of reworking existing vines which are not severely infected. These methods include layering, cutting the trunk 10 - 20 cm below any sign of infection and then training up a healthy shoot from the base of the trunk and removal of the infected trunk when the new shoots start producing and grafting.

2.4.2. Chemical control

Of the various wound protectants tested, benomyl was the most effective fungicide in protecting apricots from infection by *E. lata* (Moller and Carter, 1969). Moller *et al.* (1977) subsequently reported benomyl (Benlate®) to be more effective than wound

sealants in protecting pruning wounds on apricots from infection by *E. lata*. Further research confirmed benomyl to be effective in preventing infection by *E. lata* of apricot and grape pruning wounds (Moller and Kasimatis, 1980; Pearson, 1982; Gendloff *et al.*, 1983). Benomyl was the industry standard for prevention of eutypa dieback in grapevines until 2001, when it was withdrawn from the market by the manufacturer (E. I. Du Pont de Nemours & Co.). Munkvold and Marois (1993b) tested a range of chemicals against eutypa dieback and found flusilazole (Nustar®) to be effective. Significant reduction in infection was reported when grapevine pruning wounds were treated with NECTEC R™, a paste containing 1% propiconazole plus 2% imazilil and inoculated with a mycelial slurry of *E. lata* the day after treatment (Irelan *et al.*, 1999). However, none of the above chemicals are registered for control of eutypa dieback in Australia.

2.4.3. Biological control

2.4.3.1. The need for biological control

Biological control is defined as "the reduction of inoculum density or disease - producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonists or by mass introduction of one or more antagonists" (Baker and Cook, 1974).

Interest in biological control as a viable practice in modern agriculture has accelerated in recent years. There is an increase in public awareness of the potential ecological and health hazards posed by the use of pesticides (Burge, 1988). The emergence of fungicide

resistance in pathogen populations and the non-durable nature of fungicides throughout the susceptible period of the wounds (Munkvold and Marois, 1994) have further encouraged the quest for alternative bio-control methods. Furthermore, there are no chemicals registered currently for the control of eutypa dieback of grapevines and cultural practices are only partially effective in controlling the disease (Munkvold *et al.*, 1994). Hence, biological control may be an effective component of an integrated management strategy for this destructive disease.

2.4.3.2. Microbes tested against eutypa dieback

Biological control of eutypa dieback was first achieved by treating pruning wounds in apricot trees with macro-conidia of *F. lateritium* (Carter, 1971; Carter and Price, 1974; Carter and Price, 1975). The mode of action was mainly inhibition by antibiosis, attributed to a non-volatile diffusible metabolite produced in amounts proportional to the age of the colonies (Carter and Price, 1974). Very few authors have reported mycoparasitic activity of *F. lateritium* on *E. lata*. However, Vajna (1986) observed *F. lateritium* to parasitise *E. lata* in dual cultures. According to Irelan *et al.* (1999), the Australian strain of *F. lateritium* which was benomyl resistant did not perform well in the control of *E. lata* in experiments conducted by another research group in a different geographic location. Irelan *et al.* (1999) suggested a thorough screening of biocontrol agents for uniformity of activity against a range of *E. lata* isolates to ensure reliable disease control.

Ferreira *et al.* (1991) demonstrated effective control of *E. lata,* both in the field and *in vitro*, using a strain of *Bacillus subtilis* and suggested antibiosis as the mode of

antagonism. *Cladosporium herbarum* and *F. lateritium* inhibited infection by *E. lata* in the vineyard when applied to pruning wounds (Munkvold and Marois, 1993a). A French group has suggested that microbes which naturally colonise grapevine wounds at moderate temperatures, notably a *Rhodotorula* sp., may reduce infection of *E. lata* by competition (Chapius *et al.*, 1998).

Biological control of eutypa dieback may be a cost-effective and environmentally friendly approach to disease management. However, while control of eutypa dieback using other microbes has been achieved at the research level none have been commercially developed and evaluated in commercial situations.

2.4.3.3. **Trichoderma spp.** ***in biological control***

Trichoderma spp. have been used extensively as biological control agents for diseases in many different crops, including cotton, lettuce, onions, peas, grapes, plums and apples, caused by pathogens such as *Pythium, Phytophthora, Rhizoctonia, Fusarium, Sclerotinia* and *Botrytis* spp. (Sivan and Chet, 1989; Nelson, 1991). *T. harzianum*, *T. viride* and *T. hamatum* have been used in the biological control of the various plant pathogens mentioned above. A preparation of *T. hamatum* comprising a wheat-bran/peat mixture controlled disease caused by *Pythium aphanidermatum* in pea, cucumber and tomato, and by *R. solani* and *Sclerotium rolfsii* in beans in the glasshouse (Sivan and Chet, 1982). Sivan *et al.* (1984) also reported control of *P. aphanidermatum* using an isolate of *T. harzianum*. Control of fruit rot of tomato caused by *R. solani* has been demonstrated using *T. harzianum* in field conditions (Strashnov *et al.*, 1985). Conidia of *T. harzianum* prevented co-inoculated conidia of *Botrytis cinerea* from infecting newly

opened strawberry flowers in laboratory, glasshouse and field conditions (Hjeljord *et al.*, 2001). *T. harzianum* has been successfully used against *B. cinerea*, a serious pathogen on grapevines, under conditions of low disease incidence (Elad, 1994; Harman *et al.*, 1996). Dry rot of apples caused by natural infection of *B. cinerea* was controlled by *T. harzianum* (Tronsmo and Ystass, 1980). *T. harzianum*, *T. longibrachiatum* and *T. stromaticum* were reported to reduce witches' broom disease while *T. virens* was shown to reduce black pod disease in cocoa (Krauss and Soberanis, 2002).

Trichoderma spp. have been used successfully in curative and prophylactic treatments for various diseases in tree crops. Silver leaf disease of peach caused by *Chondrostereum purpureum* was cured when *T. viride* was introduced into the trees in a paste of glycerol and barley flour, or as a spore suspension (Dubos and Ricard, 1974). *Trichoderma* spp. introduced to freshly-cut stumps of *Eucalyptus diversicolor* prevented infection by *Armillaria luteobubalina* (Nelson *et al.*, 1995). Infection of wounds on red maple by *Fomes connatus* was reduced when the fresh wounds were painted with chopped *T. harzianum* hyphae in glycerol (Smith *et al.*, 1981).

Commercial preparations of *Trichoderma* spp. are now available. *T. virens* is available as GlioGard™ for the control of seedling diseases of ornamental and bedding plants, while *T. hazianum* is sold as F- stop™ to control several soil borne plant pathogenic fungi. BINAB T™ is a commercial product containing *T. harzianum* and *T. polysporum,* available to control wood decay (Samuel, 1996; Agrios, 1997). A New Zealand-based company, Agrimm Technologies Ltd, has formulated a range of biological products containing *T. harzianum.* Trichoseal® and Trichospray® are pruning wound applications, which are designed to control wood decay fungi. Other products

such as Trichoject® and Trichodowels® are *Trichoderma* formulations registered with the New Zealand Pesticides Board for use in the control of silver leaf (*Chondrostereum purpureum*) on pip and stone fruit trees and *Armillaria* root rots of kiwi fruit. The manufacturer suggests that these products could also be used to control eutypa dieback of grapevines (Hunt, 1999), and a New Zealand registration has recently been approved (J. Hunt, pers. com.).

2.4.3.4. Mechanisms of antagonism by **Trichoderma** *spp.*

The three major mechanisms proposed to explain antagonistic interactions between *Trichoderma* spp. and other fungi are antibiosis, mycoparasitism and competition (Hjeljord and Tronsmo, 1998).

The various metabolites produced by *Trichoderma* spp. were found to inhibit many plant pathogens. Dennis and Webster (1971a; 1971b) found that many isolates of *Trichoderma* produced volatile and non-volatile antibiotics. The metabolites produced by *Trichoderma* spp. are classified as alkyl pyrones, isonitriles, polyketides, peptaibols, diketopiperazines, sequiterpenes and steroids (Ghisalberti and Sivasithamparam, 1991; Howell, 1998). The coconut-scented compound 6-pentyl-pyrone, which belongs to the group of alkyl pyrones, was first identified in isolates of *T. viride* (Collins and Halim, 1972). Bisby (1939) previously noted this peculiar odour from cultures of *T. viride*. 6-pentyl-pyrone has now been isolated also from *T. harzianum*, *T. koningii* and *T. harmatum* (Simon *et al.*, 1988; Ghisalberti and Sivasithamparam, 1991). This volatile metabolite shows antibiotic activity against several plant pathogens (Claydon *et al.*, 1987; Ghisalberti *et al.*, 1990). Various isonitriles have been isolated from *T. harzianum*,

T. hamatum, T. koningii, T. polysporum and *T. viride* (Fujiwara *et al.*, 1982). Isonitrin A is effective against both bacteria and fungi while isonitrin D showed activity against fungi only. Harzianolide, which belongs to the polyketide group and was isolated from *T. harzianum*, inhibited growth of the take-all fungus (*Gaeumannomyces graminis*) and suppressed take-all disease of wheat in the glasshouse (Almassi *et al.* 1991). *T. koningii* has also been reported to produce harzianolide (Dunlop *et al.*, 1989).

Non-volatile, chloroform-soluble antibiotics such as trichodermin and dermadine, and peptide antibiotics, such as alamethicine and suzukacilline, which were initially extracted from cultures of *T. viride*, were found to be active against a range of fungi and bacteria (Dennis and Webster 1971a). The sesquiterpene metabolite, heptelidic acid, which was isolated from *T. viride, T. virens* and *T. koningii*, showed antibiotic activity against *P. ultimum* and *R. solani* (Howell *et al.*, 1993). The steroid, viridin, first isolated from *T. viride* (Brian and McGowan, 1945) was reported to inhibit germination of spores of many fungi (Brian and Hemming, 1945). Viridin was also reported to inhibit growth of *R. solani* and *P. ultimum* and germination of sclerotia of *Sclerotium rolfsii* (Lumsden *et al.*, 1992b). Gliotoxin and gliovirin produced by *T. virens* have been implicated in the biocontrol of soil borne fungi such as *R. solani*, *Phytophthora* spp. and *Pythium ultimum* (Weindling, 1941; Wright, 1952; Howell and Stipanovic, 1983; Lumsden *et al.*, 1992a; Wilcox *et al.*, 1992; Howell *et al.*, 1993). It is clear from these reports that the metabolites produced by *T. harzianum* have an antagonistic effect on many plant pathogens. However, reports on the effects of the metabolites produced by *T. harzianum* on *E. lata* are scarce. Hence, it would be useful to determine if the *T. harzianum* strains used in this study produce antibiotics that may inhibit *E. lata*.

Many authors have cited mycoparasitism as a mechanism of antagonism by *Trichoderma* spp. (Boosalis, 1964; Harman *et al*., 1980; Chet and Baker, 1981; Elad *et al*., 1983c). Mycoparasitic activity has been commonly observed in *T. harzianum*, *T. hamatum* and *T. koningii*. Some destructive soft rot and wilt pathogens of vegetables and field crops, such as *S. rolfsii, R. solani* and *S. sclerotiorum*, are parasitised by *Trichoderma* spp. *Trichoderma* spp. were observed to parasitise *Phytophthora cinnamomi* (Elad *et al*., 1983c; Trutman and Keane, 1990; Chambers and Scott, 1995). Mycoparasitism of *R. solani* by *Trichoderma* spp. was observed by Elad *et al*. (1983c).

Mycoparasitism generally involves four successive stages. (1) Chemotropic growth of antagonist towards the host fungus (Chet *et al*., 1981). (2) Recognition between host and the antagonist. Lectins, which are sugar-binding proteins, have been shown to be involved in the host-mycoparasite relationship between *T. harzianum* and *Rhizoctonia solani* (Elad *et al*., 1983a). Specific lectin activity has been reported between *Sclerotium rolfsii* and conidia of *T. hamatum* T-244 (Barak *et al*., 1985). (3) Attachment and coiling of hyphae of the antagonist around the host. *Trichoderma* hyphae usually attach to the host by forming appressoria or hook-like structures (Harman *et al*., 1981; Elad *et al*., 1983a; Inbar *et al*., 1996). (4) Penetration of host hyphae by the mycoparasite. *Trichoderma* penetrates by secreting lytic enzymes, which degrade the cell walls of the host fungus (Hadar *et al*., 1979a; Hadar *et al*., 1979b; Chet and Baker, 1981; Elad *et al*., 1983c; Baker, 1987). Chitinase, glucanase and protease are the main enzymes involved in degradation of fungal cell walls (Elad *et al*., 1982; Inbar and Chet, 1995).

Mycoparsitism is an important phenomenon in biological control. However, little information is available on parasitic interactions between *T. harzianum* and *E. lata* and

the relative importance of mycoparsitism in the vine. Therefore, it is necessary to investigate this aspect of biological control.

Competition is an important aspect of biological control. It occurs when the pathogen and the antagonist demand more of the same resource than is immediately available. Disease control may be achieved if the growth of the antagonist results in reduction of the pathogen population or inoculum production (Hjeljord and Tronsmo, 1998). *Trichoderma* spp. are considered to be aggressive competitors because of their rapid rate of growth, prolific conidiation and the ability to utilise a range of substrates. Biological control by competition by *Trichoderma* spp. to control fungi such as *Botrytis cinerea* and *Sclerotinia* spp. in apple, strawberry and cucumber has been demonstrated (Tronsmo and Dennis, 1977; Tronsmo and Raa, 1977; Elad *et al.*, 1993). These pathogens opportunistically invade dead or senescing plant tissues as a nutrient base before colonising healthy tissues. *Trichoderma* spp. sprayed on grape flowers during blossom delay colonisation by *B. cinerea* and, thereby, reduce disease levels in the berries (Dubos, 1987b; Harman *et al.*, 1996). Seed treatment of cereal and vegetable crops with *Trichoderma* spp. reduced germination of sporangia of *P. ultimum* by competing for the root exudates that stimulate germination of the pathogen (Ahmad and Baker, 1988; Harman and Nelson, 1994). Early colonisation of fresh wound sites by *T. viride* applied in sprays or via pruning shears has been used to control *C. purpureum* (Grosclaude *et al.*, 1973; Corke, 1974). Application of *T. harzianum* to grapevine pruning wounds may, therefore, prove useful in preventing infection by *E. lata*.

2.5. Other trunk diseases

Other major trunk diseases of grapevine include esca and Petri disease (formerly called black goo decline). The occurrence of these two diseases was confirmed in Australia only recently (Pascoe, 1999). Esca is a complex disease of grapevine, caused by wood rotting basidiomycetes in association with *Phaeomoniella chlamydospora* (formerly known as *Phaeaoacremonium chlamydosporum*). Symptoms of esca develop due to interactions between several factors (Mugnai *et al.*, 1999). It has been suggested that esca is caused by a succession of fungi in diseased vines following initial colonisation by *E. lata* and *Cephalosporium* sp. (Chiarappa, 2000). Three species of fungi, *Fomitiporia punctata*, *P. chlamydospora* and *P. aleophilum* are thought to be associated with esca of grapevines, the latter two being suspected of acting as precursors for wood decay (Larignon and Dubos, 1997). However, Sparapano *et al.* (2000) demonstrated that *F. punctata* alone colonised the wood and caused esca in grapevines. Esca symptoms include "apoplexy", "tiger stripe" leaf symptoms and black blotches on fruit widely known as "black measles" symptoms. Affected cordons show a soft, creamy, white heart rot. *P. chlamydosporum* is always found surrounding the heart rot (Pascoe, 1999). Although esca is currently not a major disease in Australia, the incidence and severity of esca may increase in the next few decades since *P. chlamydosporum* has been commonly observed in newly planted vines (Pascoe and Cottral, 2000).

Petri disease affects both young and mature vines, but decline is rapid in young vines that may show stunting and produce chlorotic leaves and thin stems. Wood symptoms involve black streaks in the heartwood and a glistening black exudate from cut vessels (Pascoe, 1999). Petri disease may occur in newly planted vineyards and is caused by

Phaeomoniella chlamydospora (Morton, 2000; Pascoe and Cottral, 2000). These diseases need to be taken into account in studies of the efficacy of biological control.

2.6. Summary

Eutypa dieback, which is a global problem, affects older vineyards and the grape industry worldwide faces the risk of losing vineyards to this disease. The pathogen enters the vines through large pruning wounds. Symptoms may not be detected immediately as the pathogen proliferates very slowly in the host tissues. The foliar symptoms are thought to be due to toxins produced by *E. lata* in the wood and translocated to the foliage. Diseased vines show reduced vigour and declining productivity, and eventually die. Disease development and dissemination are influenced by environmental factors such as rainfall, temperature and relative humidity.

The conventional methods discussed in this review are inadequate to manage this destructive disease. Hence, biological control may be a useful component of an effective disease control strategy. Very little is known about interactions between *Trichoderma* spp. and *Eutypa lata* in grapevine wood. It is imperative that the interactions between the pathogen and the antagonists are thoroughly investigated and well understood in order to formulate an integrated pest management strategy, comprising conventional and biological control methods, to ensure the long-term sustainability of grape and wine production.

CHAPTER 3. GENERAL MATERIALS AND METHODS

The materials and methods described in this chapter are those which have been used often in the experiments which are described in detail in the relevant chapters.

3.1. The host

Various cultivars of grapevine were used in these investigations. Shiraz was used in all laboratory experiments while both Shiraz and Chardonnay were used in the glasshouse experiments, except in the preliminary investigations. The field trials involved older cultivars such as Exotic, Ribier, Nyora, Rondella and Palomino, as well as the contemporary cultivars Shiraz and Cabernet Sauvignon. Exotic, Ribier and Nyora were grown in the Alverstoke vineyard, Waite Campus; these vines were considered expendable by the vineyard manager. Rondella and Palomino were grown in an abandoned vineyard in Warriparinga, close to the Flinders University of South Australia, while Shiraz was grown in a commercial vineyard at Eden Valley and Cabernet Sauvignon was grown in research vineyards at Nuriootpa, South Australia. The cultivars were assigned to the various experiments, depending on their availability, as given in Table 3.1.

In the glasshouse trials, 1-year-old cuttings of cultivars Chardonnay and Shiraz obtained from the Riverland Vine Improvement Committee, Barmera, South Australia were used. The cuttings were pretreated with hot water at 50 ± 1°C for 30 min. These two-node cuttings were allowed to stand in tap water overnight after the third basal bud was removed, before they were subjected to the various treatments, described in Chapter

5. These cuttings were inserted into rockwool pieces (4 x 4 cm Grodan blocks, supplied by Home Hydro, Glen Osmond, South Australia) which were saturated with water, and arranged on beds of vermiculite in trays (29 x 34 cm). The cuttings were maintained in the glasshouse in natural light at 5-35°C and were watered using tap water every other day. The cuttings in rockwool pieces were transferred to pots (20 cm diam.) filled with University of California (UC) potting mix (Baker, 1957) after 6 to 8 weeks, in the same layout as in the trays. These transfers were necessary to accommodate vigorous vine growth in the glasshouse. In glasshouse experiments 4, 5, 6 and 7, the cuttings inserted into the rockwool pieces were placed directly into UC potting mix in 20 cm diam. pots. The water-saturated rockwool pieces provided sufficient moisture for root intiation. Tap water was used to water the plants, every 2 days, and the cuttings were treated with Thrive™ nutrient solution (Arthur Yates and Co. Ltd; Homebush, NSW, Australia) every 6 weeks. The cuttings were maintained in the glasshouse as above. The 1-year-old cuttings used in all the experiments were 10-20 mm in diameter.

3.2. The pathogen

3.2.1. Isolates of *E. lata*

The isolates of *E. lata* which were used in experiments are listed in Table 3.2. The cultures were incubated at 22 to 25°C in darkness for 5 to 7 days and mycelial plugs were obtained from the margins of the colonies (Carter, 1991).

3.2.2. Extraction of ascospores

Pieces of infected dead wood (5–10 cm long) with stromata of *E. lata* were washed in tap water and then soaked in Reverse Osmotic (RO) water for 1 h (Carter, 1991). They were transferred to 70 mm wide and 145 mm deep sterile polycarbonate tubs with polypropylene lids (Magenta^{TM}, GA7) and allowed to discharge ascospores overnight. The ascospores discharged from the perithecia were collected in 1 ml sterile distilled water (SDW) and serially diluted to the desired concentration. Spores were counted using a light microscope (Leitz wetzlar, Orthoplan 871288) with the aid of a haemocytometer (Improved Neubauer B. S. 748).

3.2.3. Viability testing of ascospores

Viability was tested when ascospores were used as inoculum in the experiments. The spore suspensions prepared as described in section 3.2.2 were diluted to 10^3 spores/ml and 0.1 ml of this suspension was pipetted on to potato dextrose agar (PDA, Difco) or 2% water agar (WA, Bitek Agar, Difco) plates. These plates were incubated at 23°C in the dark for 2-4 days and germinating spores were counted by examining the plates in natural light. The percentage of germination was found to be 100% in all instances.

TABLE 3.1. List of grapevine cultivars used in this project.

Type of experiment	Experiment	Cultivar	Age
Laboratory	Test 1	Shiraz	1-yr-old canes
Laboratory	Test 2	Shiraz	1-yr-old canes
Laboratory	Test 3	Shiraz	1-yr-old canes
Glasshouse	Experiment 1	Shiraz	1-yr-old canes
Glasshouse	Experiment 2	Chardonnay, Shiraz	1-yr-old canes
Glasshouse	Experiment 3	Chardonnay	1-yr-old canes
Glasshouse	Experiment 4	Shiraz	1-yr-old canes
Glasshouse	Experiment 5	Shiraz	1-yr-old canes
Glasshouse	Experiment 6	Shiraz	1-yr-old canes
Glasshouse	Experiment 7	Chardonnay	1 yr-old canes
Field	Nuriootpa 1[a]	Cabernet Sauvignon	23 yr-old vines
Field	Nuriootpa 2[a]	Cabernet Sauvignon	16-yr-old vines
Field	Eden Valley[a]	Shiraz	27-yr-old vines
Field	Warriparinga 1[b]	Rondella	> 60-yr-old vines
Field	Warriparinga 2[b]	Palomino	> 60-yr-old vines
Field	Trichodowel trial[b]	Nyora	22-yr-old vines
Field	Injection trial[b]	Ribier, Exotic	22-yr-old vines

[a] *Pruned routinely using the "Finger and thumb" method (T. Gherlach, pers. com., 2000).*

[b] *Spur pruned routinely.*

TABLE 3.2. List of isolates of *E. lata.*

Strain	Source	Origin
M280[a,b]	Grapevine wood	Victoria
M302[a]	Grapevine wood	Victoria
M295[a]	Grapevine wood	South Australia
CS-Ba.1.99/12/06	Grapevine wood (Cabernet sauvignon)	South Australia
CS-Ba.2.99/12/06	Grapevine wood (Cabernet sauvignon)	South Australia
CS-Ba.3.99/12/06	Ascospores (Cabernet sauvignon)	South Australia

[a] *Strain from F. M. Cole, Monash University, Victoria.*

[b] *Designated virulent on the basis of pathogenicity to micropropagated grapevines in vitro (M. Cole, pers. com.).*

3.3. The antagonists

3.3.1. Strains

Three strains of *T. harzianum* provided by Agrimm Technologies Ltd were used in the initial experiments to ascertain the mechanisms of antagonism. These are designated strains 1, 2 and 3 in this thesis, but have since been re-named AG1, AG2 and AG3 by the manufacturer. Strain 1 was used in all subsequent experiments on the recommendation of Agrimm Technologies Ltd. Also, *F. lateritium* was used in some experiments (Carter, 1983). The isolates were cultured on PDA or 1/6 strength Czapek Dox agar (CDA,

Difco) at 22-23°C in the dark. Mycelial plugs were taken from the margins of 3-4-day-old cultures.

Spore suspensions were prepared using 5-7-day-old cultures. The culture plates were flooded with SDW, gently scrubbed with a sterile wire-loop, transferred to a sterile polycarbonate container and the concentration of the spore suspension was ascertained with the aid of a haemocytometer (Improved Neubauer B. S. 748) and the light microscope (Leitz wetzlar, Orthoplan 871288). The spore suspension was diluted with SDW to obtain a concentration of 10^9 spores/ml in the case of *T. harzianum* or 10^6 spores/ml for *F. lateritium*.

3.3.2. Commercial formulations

The commercial formulations supplied by Agrimm Technologies Ltd contained seven strains of *T. harzianum*, including the three strains mentioned in section 3.3.1. The different types of formulations used in the glasshouse and field investigations and the experiments in which they were used are listed in Table 3.3.

3.3.3. Enumeration of colony forming units (CFU)

Malt agar (MA) was prepared by dissolving 50 g of malt extract agar (Oxoid) in 1 L of RO water and autoclaving at 121°C for 20 min. The preparation of sterile diluent, used to suspend the commercial Trichoseal® or Vinevax® formulation and to prepare the dilution series of the suspended product, is described below. The method of enumeration, based on that provided by Agrimm Technologies Ltd, is also given below.

A. Preparation of sterile diluent:

Bitek agar (Difco, 0.2 g) was dissolved in 2 L of RO water and autoclaved at 121°C for 20 min. Tween 20 (Sigma, Polyoxyethylene Sorbitan Monolaurate) and concentrated HCl, 50 μl of each, were added to each litre of the autoclaved agar solution. A series of dilutions was prepared aseptically as described in the Appendix 1.

B. Plating:

A 250 μl droplet of each dilution of each commercial formulation was applied to each of three plates of MA and spread out by gently rolling the plates from side to side. The plates were sealed with Parafilm® and incubated at 23°C for 72 h. Individual colonies on the plates were counted by examining them under natural light. The cfu/g was calculated as shown below.

C. Conversion into CFU/g:

1. The mean numbers of colonies on the replicate plates were calculated for each dilution.
2. CFU/ml for each dilution = mean of each dilution which was multiplied by 4.
3. The values obtained at step 2 were multiplied by the corresponding dilution factor for each dilution.
4. The values obtained at step 3 were multiplied by 5×10^3 to obtain the number of CFU/g of product (J. Hunt, pers. com., 1999).

The CFU assay was carried out twice to confirm that Trichoseal spray® and Vinevax® yielded CFU of *T. harzianum*, as stated by the manufacturer. The CFU/g values obtained for Trichoseal spray® and Vinevax® were 1×10^{10} CFU/g and 32×10^8 CFU/g,

respectively. The figure stated on the label for Trichoseal spray® was "at least 11 X 10^6 CFU/g" and for Vinevax® "not less than of 5 x 10^8 cfu/g".

Table 3.3. List of commercial formulations

Formulations	Strains	Experiments involved
Trichoseal®	7 strains of *T. harzianum*	Glasshouse trials 4, 5 and 6
Trichoseal spray® or *Vinevax®	7 strains of *T. harzianum*	Pruning wound trials in the field
Trichodowels®	7 strains of *T. harzianum*	Trichodowel trial
Trichoject®	7 strains of *T. harzianum*	Injection trial

** Vinevax® was marketed by Agrimm Technologies Ltd in 2002 to be used in place of Trichoseal® and Trichoseal spray®. This formulation was used only in the Warriparinga 2 Trial.*

3.4. Re-isolation of fungi from wood

3.4.1. Re-isolation of pathogen

The bark was stripped from the canes and the 1-2 cm cane segments were surface sterilised in 2.5% sodium hypochloride (NaOCl) containing a drop of Tween 80 (Sigma, Polyoxyethylene Sorbitan Monooleate) on a rotary shaker for 12 min. and washed twice with SDW. The cane pieces were then split longitudinally and cut into 5-10 mm chips. The chips were transferred to a medium selective for *E. lata* (EUSM), the composition of which is described in the Appendix 2. The plates were incubated in the dark at 23°C for 7-14 days. If any one of the chips yielded *E. lata*, the cane was considered to be infected.

E. lata mycelium was identified by comparing the colour and colony morphology with that of a known culture of similar age (Petzoldt *et al.,* 1981; Carter, 1991; Munkvold and Marois, 1993). When cultures were further incubated for 4-8 weeks, some isolates produced pale yellow or orange droplets, which contained the characteristic conidiomata with curved conidia (Carter, 1991). However, not all isolates developed conidiomata.

3.4.2. Re-isolation of antagonists

The canes were surface sterilised and cultured as described in section 3.4.1. However, the wood chips were either plated on acidified PDA (APDA) or CDA plates (see Appendix 2) and incubated at 23°C in the dark for 3-7 days to re-isolate *T. harzianum*. In the case of wood shavings collected by drilling into the trunks of vines, as in the case of the Trichodowel trial, the shavings were only washed thrice in SDW and plated on APDA in clumps. Re-isolation of *T. harzianum* was also carried out on EUSM in the simultaneous testing of *E. lata* and *T. harzianum*, when separate re-isolations were not possible due to limited availability of wood tissue. Re-isolation of *F. lateritium* was carried out as for re-isolation of *T. harzianum* from wood chips, and the chips were plated on CDA medium (see Appendix 2) and incubated at 23°C in darkness for 3-7 days. *T. harzianum* and *F. lateritium* could be identified by their colony morphology on culture plates.

3.5. Maintenance of isolates and cultures

All fungal isolates were stored at 5°C as culture plates. *T. harzianum* and *E. lata* were stored on PDA while *F. lateritium* was stored on CDA. Long-term storage of *E. lata* isolates was in SDW as mycelial plugs in McCartney bottles at room temperature (Boesewinkle, 1976; Carter, 1991).

3.6. Statistical analysis

All analyses were performed in GENSTAT for windows, 5th edition (Lawes Agricultural Trust, Rothamsted, England). Analysis of variance (ANOVA) was carried out to determine the significant differences between the treatments. LSD values at the 5% probability level were used to separate the means. For data comprising the presence or absence response, ANOVA techniques were not appropriate since the data were not normally distributed. Hence, such data were modeled using one form of generalised linear model (GLM), assuming a binomial distribution and a logit link as advised by Ms Lorimer, BiometricsSA. This method is also known as logistic regression (McCullagh and Nedler, 1989).

CHAPTER 4. INTERACTION STUDIES *IN VITRO*

4.1. Introduction

The high degree of ecological adaptability shown by *Trichoderma* spp. and their amenability to cultivation on inexpensive substrates make these fungi attractive candidates for applications in biological control. To become successful biological control agents, the isolates of *Trichoderma* not only must show ecological adaptability but must also have suitable antagonistic characteristics against the specific pathogen. According to one "school of thought", isolates of potential bio-protectants need to be screened in the laboratory before applications are tested in the glasshouse or field. However, some researchers prefer to screen potential biological control agents first in pot or field trials and, if the antagonists perform efficiently in these conditions, then mechanisms of inhibition and relevant *in vitro* studies are carried out in the laboratory (Blakeman, 1988; Faull, 1988). Also, the mechanism(s) of biological control has been used to selectively isolate potential biological control agents (Cook *et al.*, 1997). In this project, laboratory experiments were first carried out to investigate mechanism/s of inhibition of *E. lata* by *T. harzianum.*

Antagonistic interactions between *Trichoderma* spp. and other fungi have been classsified into three main categories: antibiosis, mycoparasitism and competition (see section 2.4.3.3). These mechanisms are not mutually exclusive and antagonistic activity of a biocontrol agent could fall into one or more of these categories. Biological control of *B. cinerea* on grapes was achieved by *T. harzianum* through mycoparasitism and

competition for nutrients (Dubos, 1987b). Antibiotics and hydrolytic enzymes that are produced together by *Trichoderma* spp. have been shown to act synergistically in mycoparasitism (Di Pietro *et al.*, 1993; Schirmböck *et al.*, 1994). Moreover, some antagonistic interactions may not fall into any of the three classical categories. For example, *T. harzianum* indirectly controlled the decay fungus, *Fomes connatus*, in red maple by replacing the pioneer fungus *Phialophora melinii* which renders the wood susceptible to the decay fungus by reducing the phenolic constituents of maple sapwood (Smith *et al.*, 1981). More recently, Zimmand *et al.* (1996) have suggested that *T. harzianum* T39 antagonises *B. cinerea* by reducing the amount of pectin-degrading enzymes produced by the pathogen.

The antimicrobial compounds produced by *Trichoderma* spp. constitute a diverse group of secondary metabolites with respect to structure and function. These metabolites comprise both volatile and non-volatile compounds (Dennis and Webster, 1971a; Dennis and Webster, 1971b), as described in section 2.4.3.4.

Mycoparasitism, the direct attack of one fungus on another, usually results in death of the host fungus if *Trichoderma* spp. are used as the antagonist (Barnett and Binder, 1973). Biological control due to mycoparasitism by *Trichoderma* spp. is reviewed in section 2.4.3.4.

Competition occurs when two or more microorganisms demand more of the same resource than is immediately available (see section 2.4.3.3). The three major mechanisms of antagonism are reviewed in section 2.4.3.3.

The investigations reported in this chapter were directed towards studying interactions between *E. lata* and *T. harzianum* in the laboratory on agar medium and in sterilised

cane segments to ascertain the mechanism(s) of inhibition. Scanning electron microscopy was used to examine interactions in gamma-irradiated cane segments and living canes.

4.2. Materials and Methods

4.2.1. Mode of inhibition

4.2.1.1. Antibiosis by volatile metabolites

The method of Dennis and Webster (1971b) was used to investigate the effect of volatile antibiotics produced by strains 1, 2 and 3 of *T. harzianum* on *E. lata* isolates M280 and CS-Ba.1. Plugs of *T. harzianum* (8 mm diam.) were placed in the centre of 90 mm Petri dishes containing 20 ml PDA and incubated at 22-25°C in darkness. After 2 days, the bases of fresh plates of PDA inoculated with 8 mm diam. plugs of *E. lata* in the central position were inverted over the bases of the plates with the 2-day-old antagonists, taped with Parafilm® and incubated in the same conditions for a further 5 days. Controls consisted of plates of *E. lata* inverted over PDA plates inoculated with sterile PDA plugs. There were eight replicates per treatment combination. For each test plate, the colony diameter of *E. lata* was measured (the diam. of the plug of inoculum, 8 mm, was subtracted), and the results were analysed using ANOVA (see section 3.6).

The effect of volatile metabolites on the germination of ascospores of *E. lata* was also tested. Ascospores were extracted from wood containing stromata as described in section 3.3.2 and 20 μl droplets, each containing 500 ascospores in SDW, were placed on the surface of each PDA plate. The droplets were spread evenly over the surface of the PDA

and the bases were inverted over the bases of plates that had been inoculated 2 days previously with 8-mm-diam. plugs of the three strains of antagonists, applied individually, and sealed with Parafilm®. Controls consisted of PDA plates containing the same number of ascopores inverted over PDA plates inoculated 2 days previously with 8-mm-diam. plugs of sterile PDA and sealed with Parafilm®. After 2 days of incubation at 22-25° C in the dark, the number of germinating spores that had formed colonies was counted using a dissecting microscope (Wild®; magnification X 50). There were eight replicates per treatment combination. The ascospores were too dense to count accurately. This problem was rectified in the next experiment (section 4.2.1.2) by further diluting the ascospore suspension so that a 10 μl droplet contained only 25 ascospores. However, this experiment was not repeated with further dilutions, since there was a total inhibition of germination by the volatile metabolites produced by all three strains of the antagonist.

4.2.1.2. Antibiosis by non-volatile metabolites

This experiment was based on the modified method of Dennis and Webster (1971a) used by Chambers (1993). The effect of non-volatile metabolites produced by strains 1, 2 and 3 of *T. harzianum* on six isolates of *E. lata* was investigated (M280, M295, M302, CS-Ba.1, CS-Ba.2, CS-Ba.3).

Sterile uncoated cellophane discs (80 mm diam.; Australia Cellophane, Victoria) were placed in each 90 mm diam. Petri dish, containing 20 ml PDA. The cellophane discs were first immersed in boiling water for 10 min. and then autoclaved at 121°C for 20 min. Plugs of mycelia (8 mm diam.) of the antagonists were each cut from the edge of an actively growing culture on PDA and placed in the centre of each cellophane disc. Sterile

plugs of PDA of the same dimensions served as controls. The plugs were incubated at 22-25°C in the dark. After 2 days the plug of mycelium and agar was removed together with the cellophane, replaced by a plug of *E. lata* (8 mm diam.) and the plate incubated in the same conditions. There were eight to ten replicates per treatment combination. The colony diameters of *E. lata* isolates were measured after 4 days, and the diam. of the plug of inoculum (i.e. 8 mm) subtracted before analysis of the data. In a subsequent repeat experiment, the colony diameter of the slower growing isolates M295, M302, CS-Ba.2 and CS-Ba.3 was measured after 6 days. Plugs which showed no growth of mycelium after 2 weeks were transferred to fresh PDA plates, incubated in the same conditions for a further 2 weeks and observed for signs of mycelial growth to determine whether effects were fungistatic or fungicidal. The results were subjected to ANOVA (see section 3.6).

In addition, the effect of non-volatile metabolites produced by the three strains of *T. harzianum* on germination of ascospores of *E. lata* was investigated. The antagonist was grown from a plug of inoculum on the surface of a cellophane disc exactly as described above. After 2 days of incubation the mycelial plugs of the antagonist or PDA plugs and the cellophane were removed and the plates were inoculated with a 10 μl droplet containing 25 ascospores as described in section 4.2.1.1 and incubated in the dark at 22-25°C. After 3 to 6 days the number of germinated spores was counted as described in section 4.2.1.1. There were eight replicates per treatment combination. ANOVA was used to analyse the results (see section 3.6).

4.2.1.3. Parasitism

Parasitic interactions were investigated in dual cultures (Dennis and Webster, 1971c; Chambers and Scott, 1995). A sterile strip of uncoated cellophane (80 x 30 mm; Australia Cellophane, Victoria), prepared as described in section 4.2.1.1, was placed in the centre of each 90 mm diam. Petri-dish containing 20 ml PDA. Plugs (8 mm diam.) of *E. lata* isolate M280 or CS-Ba.1 were placed on one end of the strip, 20 mm from the edge of each plate. The controls received sterile PDA plugs. The plates were incubated at 22°C for 3 days in darkness. A plug of *T. harzianum* (8 mm diam.) was transferred to the cellophane strip 50 mm from the *E. lata* plug and incubated in the dark for 3 to 4 days. The three strains of *T. harzianum* that were tested for volatile and non-volatile metabolites were also tested for parasitism. There were five replicates per treatment combination. After 3 to 4 days, 5 x 5 mm pieces of cellophane were cut from the zone of interaction, mounted on slides, stained with ammonical congo red (Chambers and Scott, 1995) and observed using the compound microscope (Leitz Wetzlar Orthoplan; magnification x 400).

4.2.2. Interactions on cane segments

Inter-nodal grapevine cane segments of cultivar Shiraz were sterilised by autoclaving or gamma-irradiation to eliminate competition from resident organisms.

4.2.2.1. Colonisation of wood by **E. lata** *in the presence of* **T. harzianum** *- experiment 1*

This experiment was based on the method used by Mercer and Kirk (1984a) to test biological treatments for the control of decay in tree wounds. Inter-nodal segments (60 mm long) of 1-year-old canes of cultivar Shiraz were split into two halves, bark was removed, and the canes sterilised either by autoclaving at 121° C for 20 min. or by gamma irradiation. Irradiation was carried out by Steritech, Victoria, Australia at 25 kgray. Bark was removed from the canes before sterilisation. Each of the split segments was then placed in the centre of a Petri dish (90 mm diam.) lined with three layers of sterile Whatman No. 42 ashless filter paper discs (80 mm diam). The filter papers were first autoclaved at 121°C for 20 min and moistened with SDW. Plugs (5 mm diam.) of the virulent *E. lata* isolate M280 were placed on the cane segment 2-3 mm from one end and the canes were incubated at 23-25°C in the dark. After 5 days, a 5-mm diam. plug of strain 1 of *T. harzianum* (or a plug of PDA of the same dimension in controls) was placed 2-3 mm from the other end of the cane segment. *E. lata* was applied before *T. harzianum* to allow the slow growing pathogen to become established in the wood. There were eight replicates per treatment combination. The co-inoculated segments were incubated for a further 3 days in the same conditions, after which the extent of growth of *E. lata* (i.e. the distance between the inoculum plug and the hyphal front) in the presence and absence of *T. harzianum* was measured. The results were analysed using ANOVA (see section 3.6).

4.2.2.2. Colonisation of wood by **E. lata** *in the presence of* **T. harzianum** *- experiment 2*

This experiment was based on the method used by Munkvold and Marois (1993a) to identify possible biological control agents for *E. lata*. Canes of cultivar Shiraz were cut into 10 mm long segments, the bark was removed and the canes autoclaved at 121°C for 20 min. then embedded upright in 2 % water agar in 140 mm diam. Petri dishes. There were 10 segments in each of four dishes. One of the following four treatments was administered to the 10 cane segments in one of the four Petri dishes so that there was one plate of 10 segments per treatment. The plates were incubated at 23°C for 24 h in darkness.

Treatment 1 = 5 mm sterile PDA plug (control)

Treatment 2 = 10 μl droplet of SDW (control)

Treatment 3 = 5 mm plug of strain 1 of *T. harzianum*

Treatment 4 = 1,500 spores of strain 1 of *T. harzianum* in a 10 μl droplet of SDW

The treatments were applied to the upper freshly cut surface of the cane segments. After 24 h the canes were inoculated with 5 mm plugs of *E. lata* isolate M280 (see Table 3.2). The mycelial plugs of the antagonist and the plugs of PDA were removed aseptically from the canes that received Treatments 1 and 2 before these were inoculated with the pathogen. The segments were incubated for a further 10 days. Re-isolation of *E. lata* was carried out by cutting each segment into 10 chips after surface sterilisation in 2.5 % NaOCl as described in section 3.4.1. Statistical analysis was deemed unnecessary since there was complete inhibition of colonisation of canes by *E. lata* in the presence of *T. harzianum*.

4.2.2.3. Colonisation of wood by **E. lata** *in the presence of* **T. harzianum** *- experiment 3*

This experiment was based on the method of Rolshausen and Gubler (1998). Bark was removed from 1-year-old canes of cultivar Shiraz and 30 mm long segments were cut from these canes. Each segment was placed into a McCartney bottle containing 3 ml of SDW and autoclaved at 121°C for 20 min. The cane segments were treated with a 25 μl SDW droplet containing 1,000 ascospores or conidia of *E. lata* or *T. harzianum* (strain 1), respectively, in the following way. There were 10 replicates per treatment combination.

Treatment 1 = *E. lata* ascospores + *T. harzianum* (strain 1) conidia

Treatment 2 = *T. harzianum* (strain 1) conidia only (control)

Treatment 3 = *E. lata* ascospores only (control)

Treatment 1 involved application of a 25 μl droplet of spore suspension of each fungus at the same time. The inoculated segments were incubated at 23°C for 4 weeks, after which surface sterilisation and re-isolation of *E. lata* and *T. harzianum* on EUSM were carried out as described in sections 3.4.1. and 3.4.2.

4.2.3. Microscopy

Interactions between *E. lata* and *T. harzianum* were investigated in 1-year-old canes of cultivar Shiraz using the Field Emission Scanning Electron Microscope (SEM) (Philips Field Emmission Scanning Electron Microscope XL30). Initially, uninoculated canes were checked for the presence of endophytic fungi by microscopic examination

((Leitz Wetzlar Orthoplan; magnification x 400). Cane segments (1-1.5 cm long) that were sterilised by gamma-irradiation at 25 kgray, as described in section 4.2.2.1, were used. Also, interaction in living canes was studied using cuttings grown in rockwool pieces (4 x 4 cm Grodan blocks) in the laboratory. The specimens were processed for SEM as described in section 4.2.3.1.

4.2.3.1. Processing of wood for SEM

The samples were fixed overnight in SEM fixative comprising 4% paraformaldehyde; 1.25% glutaraldehyde in phosphate buffered saline (PBS, see Appendix 2); 4% sucrose; pH 7.2. Next, the samples were washed twice for 30 min. in two changes of washing buffer (PBS + 4% sucrose) and post-fixed in 1% OsO_4 in PBS for 1 h, then dehydrated through an ethanol series of 70%, 90%, 95% and 100% ethanol in water. The samples were dehydrated for 20 min. thrice at each concentration of ethanol and at the final stage of 100% ethanol, after the three changes of 20 min., dehydration was carried out for an additional 1 h. The dehydrated segments were then immersed in a 1:1 mixture of 100% ethanol and 100% acetone for 15 min. and transferred to 100% acetone for another 15 min. Thereafter, the specimens were dried in the critical point drier (Bal-tec 030), mounted on metal stubs, coated (3 nm in thickness) with gold and palladium and viewed under the microscope at an accelerating voltage of 10 kV.

4.2.3.2. Inoculation of gamma-irradiated wood

Cane segments were inoculated with a 5 mm diam. plug of *E. lata* strain M280 at one of the cut ends and incubated at 23°C in darkness in 9 mm diam. Petri-dishes lined with

sterile Whatman No. 42 ashless filter paper discs (80 mm diam). The filter papers were first sterilised by autoclaving at 121°C for 20 min. and then moistened with SDW. After 4 days, the cane pieces were inoculated with plugs (5 mm diam.) of *T. harzianum* strain 1 at the other end and incubated for a further 3 days as before. The filter paper lining was moistened again with SDW at this stage. Controls were inoculated with M280 or *T. harzianum* alone. There were three replicates per treatment combination. After 3 days the inoculated pieces were split open and processed for SEM as described in section 4.2.3.1.

4.2.3.3. Inoculation of canes grown in rockwool pieces in the laboratory

Single-node canes of cultivar Shiraz were allowed to stand in water overnight after the second basal bud was removed. The cuttings were pruned 2-4 cm above the bud and inoculated on the freshly cut surfaces with 5 mm diam. plugs of M280 or ascospores of *E. lata* (1,000 spores in a 25 μl droplet of SDW). The canes were grown in rockwool pieces in a water-saturated condition on the laboratory bench at approximately 22°C in natural light. After 2 days, the canes that had been inoculated with M280 were either inoculated with 5 mm diam. mycelial plugs of *T. harzianum* (strain 1) or with a 25 μl droplet of SDW containing 1,000 conidia of *T. harzianum* (strain 1) and the canes that had been inoculated with ascospores of *E. lata* were inoculated with conidia of strain 1. The controls were inoculated with mycelial plugs of M280 alone, ascospores of *E. lata* alone, mycelial plugs or conidia of *T. harzianum* (strain 1) alone. The inoculum was covered with Parafilm® at each stage. There were three replicates per treatment combination. The canes were harvested after 2 weeks and 1 cm long segments below the point of inoculation were excised split open and processed for SEM (section 4.2.3.4).

4.2.3.4. Simultaneous co-inoculation of canes grown in rockwool pieces in the laboratory

This was similar to the experiment described in section 4.2.3.3, except the pruning wounds were simultaneously treated with spore suspensions of *E. lata* and *T. harzianum* (strain 1) prepared in SDW, both of which were introduced in a 25 μl droplet containing 1,000 spores. The controls consisted of treatments with either ascospores of *E. lata* alone or conidia of *T. harzianum* alone. The canes were harvested after 30 min., 6 h, 22 h, 7 days, 14 days and 21 days. There were three replicates per treatment combination. Cane segments (1 cm long) below the point of inoculation were excised, split into two and processed for SEM as described in section 4.2.3.1.

4.3. Results

4.3.1. Mechanisms of inhibition *in vitro*

4.3.1.1. Inhibition by volatile metabolites

The volatile antibiotics produced by all three strains of *T. harzianum* significantly ($P<0.001$) reduced growth of both isolates of *E. lata* tested (Figure 4.1). However, there was significant variation in the extent of the growth reduction of *E. lata* in the presence of volatile metabolites produced by the three strains ($P<0.001$). For example, the volatile metabolites produced by strain 2 were the most effective in reducing the growth of isolate M280. The volatile antibiotics produced by strain 3 inhibited isolate CS-Ba.1 most strongly, while those produced by strains 1 and 2 had similar effects on this isolate.

Also, the volatile metabolites of the three strains of *T. harzianum* totally inhibited germination of ascospores of *E. lata* (Table 4.1). Incubation was carried out for 2 days as germination was assessed on the basis of colonies visible using the dissecting microscope. Incubation for more than 2 days was not attempted as two replicates of each of the *E. lata* cultures treated with strains 1 and 2 and all eight replicates treated with strain 3 were contaminated with *T. harzianum* after 2 days. A coconut odour was emitted from culture plates of the three strains of *T. harzianum*.

4.3.1.2. Inhibition by non-volatile metabolites

The three strains of *T. harzianum* significantly ($P<0.001$) inhibited growth of all six isolates of *E. lata* (Figures 4.2 and 4.3). However, total inhibition of growth was not observed for isolates M280, CS-Ba.1 and CS-Ba.2 by any of the three strains of *T. harzianum* while CS-Ba.3 was completely inhibited only by strain 1. The mycelial growth of isolates M295 and M302 was completely inhibited by the three strains of *T. harzianum* (Figure 4.3). No growth was observed even when the plugs of isolate M295 and M302 were transferred to fresh PDA medium and incubated for a further 2 weeks in the dark at 22-25°C, therefore the effect was deemed fungicidal. The non-volatile metabolites produced by strains 1, 2 and 3 had a fungistatic effect on the remaining four isolates of *E. lata*. The data (Figures 4.2 and 4.3) for the two experiments were analysed separately.

The effect of the non-volatile antibiotics produced by the three strains of the antagonist was not significantly different for isolates M280 or CS-Ba.1 (Figure 4.2). However, the non-volatile antibiotics produced by strain 1 were significantly more

effective (P<0.001) in reducing growth of isolate CS-Ba.3 than those produced by the other two strains (Figure 4.3). In the case of isolate CS-Ba.2, all three strains of *T. harzianum* were equally effective in reducing growth. These results suggest that the degree of inhibition by the three strains of *T. harzianum* may differ for a given isolate of *E. lata.*

Germination of ascospores of *E. lata* was significantly inhibited by the non-volatile metabolites produced by all three strains of *T. harzianum* (P<0.001). The non-volatile metabolites of strains 2 and 3 totally inhibited germination (Figure 4.4).

4.3.1.3. Inhibition by parasitism

There was no evidence of mycoparasitisn in dual cultures on PDA when the interaction zones of dual cultures were examined using the light microscope (Leitz Wetzlar Orthoplan). Distinguishing the hyphae of the pathogen from the antagonist was not possible, since both were of the same dimensions and showed similar branching patterns.

4.3.2. Interaction studies on cane segments

4.3.2.1. Interactions between pathogen and antagonist - experiment 1

In the absence of *T. harzianum*, *E. lata* grew an average of 42 mm along the autoclaved cane segments, significantly further than along the gamma-irradiated cane segments (38 mm). There was no difference in growth of the pathogen on autoclaved and gamma-irradiated co-inoculated canes (Figure 4.5). Growth of *E. lata* was slightly, but

significantly, retarded on both autoclaved canes and gamma-irradiated canes when mycelial plugs of *T. harzianum* were introduced 5 days after *E. lata* (P=0.023).

4.3.2.2. Interactions between pathogen and antagonist - experiment 2

E. lata colonised all of the control cane segments which had been treated with sterile PDA plugs or SDW. *E. lata* was not re-isolated from any of the autoclaved canes which had been inoculated 24 h previously with spores or mycelial plugs of *T. harzianum* (Figure 4.6).

4.3.2.3. Interactions between pathogen and antagonist - experiment 3

The pathogen and antagonist were isolated from all of the appropriate control segments (Figure 4.7). *T. harzianum* was isolated from 90% of co-inoculated canes and *E. lata* from 10% of the canes. This difference was statistically significant ($P<0.001$) according to the χ^2- test. None of the cane segments yielded both fungi when cultured on EUSM.

Figure 4.1. Inhibition of growth of two isolates of *E. lata* due to volatile antibiosis by three strains of *T. harzianum* on PDA. Mycelial plugs of *T. harzianum* were grown on PDA for 2 days in the dark at 22-25°C. Bases of fresh plates of PDA inoculated with *E. lata* were inverted over the bases of plates containing the antagonist and incubated for 5 days. Y-axis denotes the average colony diam. (minus the inoculum plug) for eight replicates per treatment combination. Bars denote standard errors.

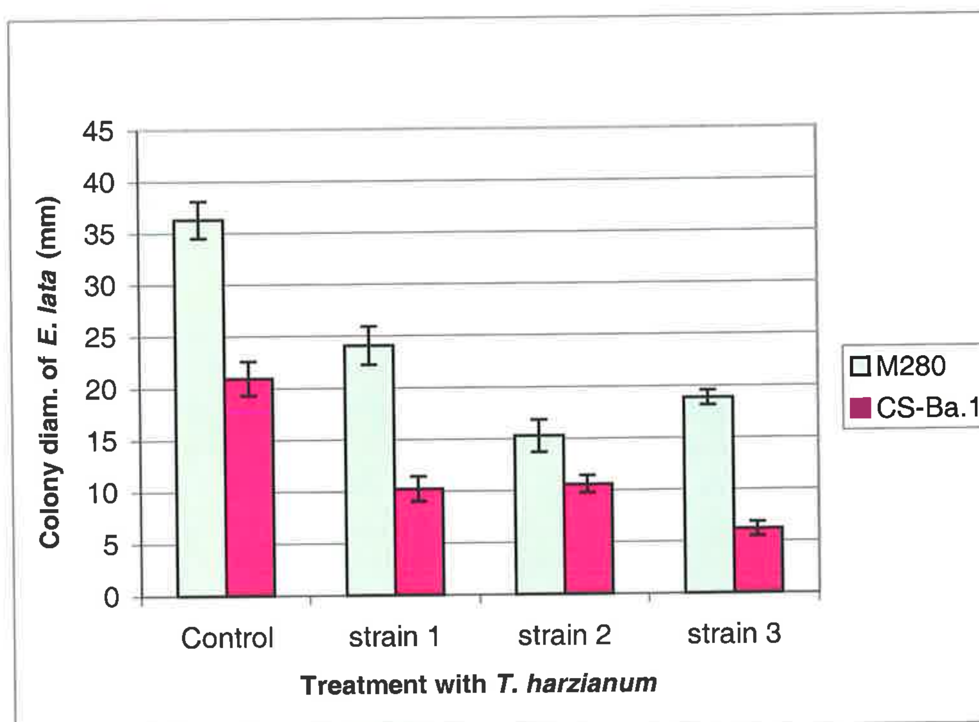


Table 4.1. Inhibition of germination of ascospores of *E. lata* by volatile metabolites produced by three strains of *T. harzianum*. Mycelial plugs of *T. harzianum* were grown on PDA for 2 days in the dark at 22-25°C. A 20 μl droplet containing 500 ascospores was spread on a fresh PDA plate and the base of the plate was inverted over the base of the plate containing the 2-day-old antagonist and incubated for another 2 days. There were eight replicates for each treatment combination.

Treatment	**Average number of germinated ascospores**
PDA plug + ascospores of *E. lata* (control)	>100[a]
Strain 1 + ascospores of *E. lata*	0
Strain 2 + ascospores of *E. lata*	0
Strain 3 + ascospores of *E. lata*	0

[a]*Estimates only, as germlings were crowded on the plate*

Figure 4.2. Inhibition of growth of two isolates of *E. lata* due to non-volatile antibiosis by three strains of *T. harzianum* on PDA. Mycelial plugs of *T. harzianum* were grown on cellophane discs on PDA for 2 days in the dark at 22-25°C. The cellophane discs and mycelial plugs were removed and replaced by plugs of *E. lata*, and plates incubated for another 4 days. Y-axis denotes the average colony diam. (minus the inoculum plug) of the eight replicates of *E. lata* receiving each treatment combination. Bars denote standard errors.

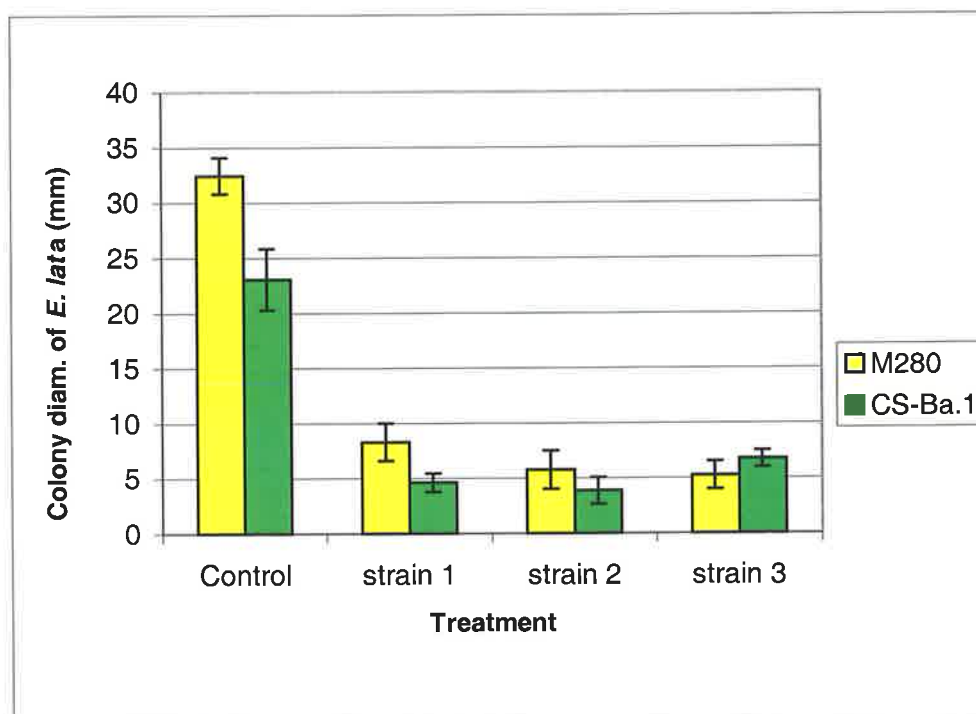


Figure 4.3. Inhibition of growth of four isolates of *E. lata* due to non-volatile antibiosis by three strains of *T. harzianum* on PDA. Mycelial plugs of *T. harzianum* were grown on cellophane discs placed on surface of PDA for 2 days in the dark at 22-25°C. The cellophane discs and mycelial plugs were removed and replaced by plugs of *E. lata*, and plates incubated for another 6 days. Y-axis denotes the average colony diam. (minus the inoculum plug) of the five replicates of *E. lata* receiving each treatment combination. Bars denote standard errors.

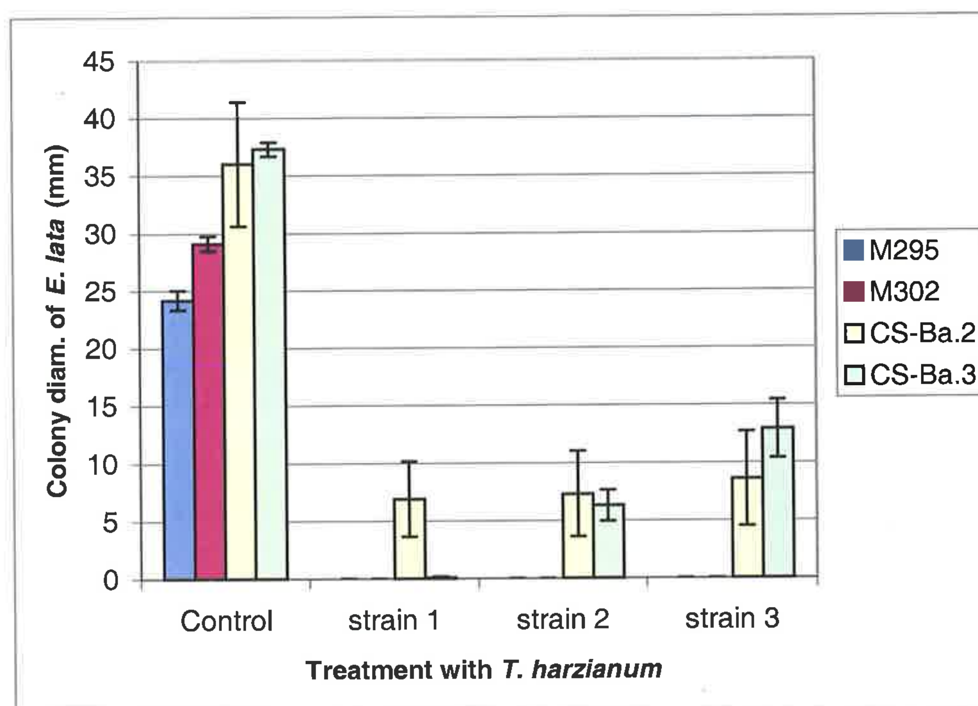


Figure 4.4. Inhibition of germination of ascospores of *E. lata* due to non-volatile metabolites produced by three strains of *T. harzianum*. Mycelial plugs of *T. harzianum* were grown on cellophane discs placed on PDA for 2 days in the dark at 22-25°C. The cellophane discs and mycelial plugs were removed and a 10 μl droplet containing 25 ascospores were spread on the PDA and incubated for another 3-6 days. Y-axis denotes the average percentage of ascospores germinated in the eight replicates per each treatment. Bars denote standard errors.

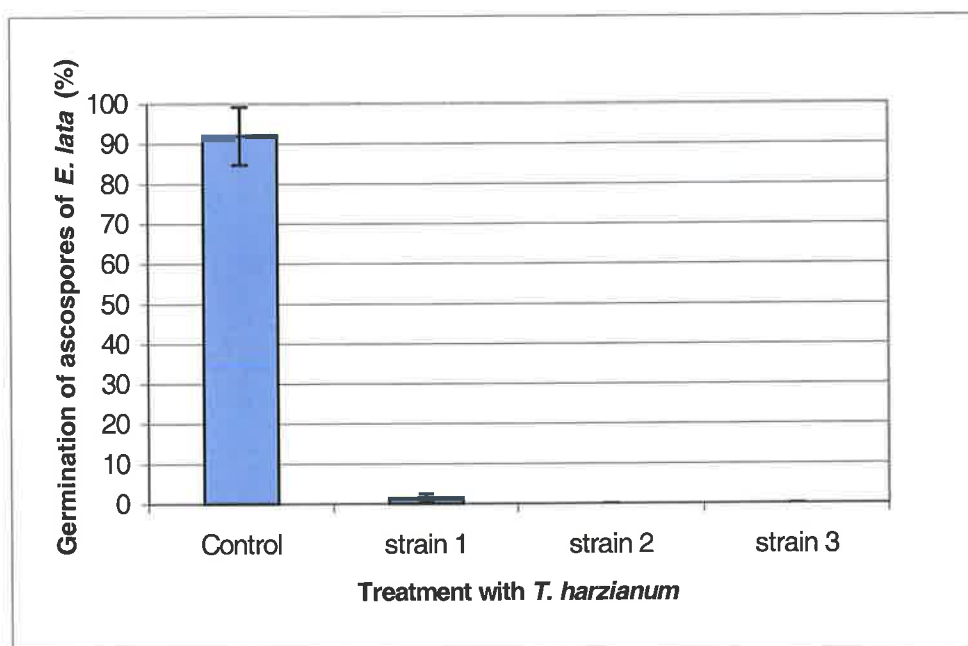
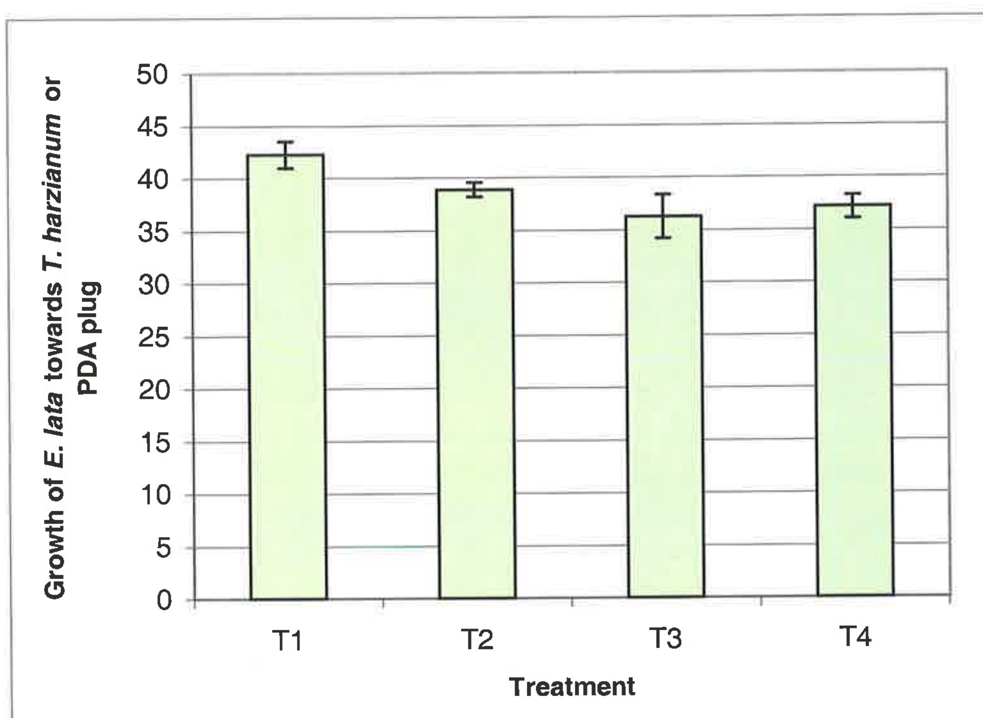


Figure 4.5. Growth of *E. lata* on sterilised canes in the presence of *T. harzianum*, based on the method of Mercer and Kirk (1984a). Sterilised cane segments of cv. Shiraz were inoculated at opposite ends with 5 mm plugs of *E. lata* and *T. harzianum* or PDA and incubated at 23-25°C in the dark. *E. lata* was applied 5 days prior to *T. harzianum*. Growth of *E. lata* was measured 3 days after the antagonist was applied. Y-axis denotes the average growth of the eight replicates of *E. lata* receiving each treatment combination. Bars denote standard errors.



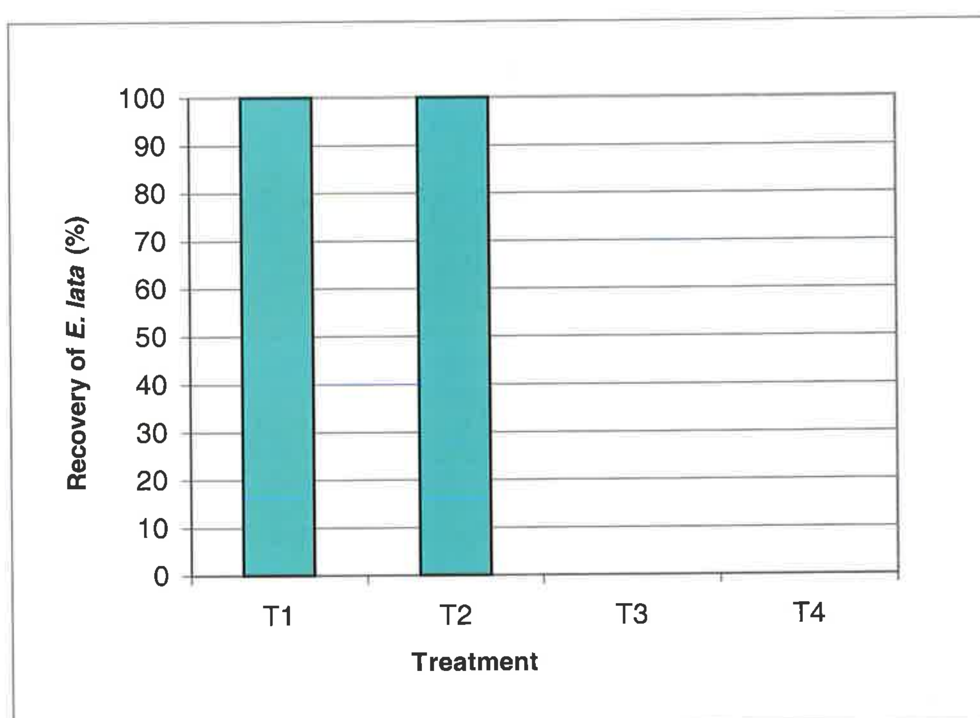
T1 = *E. lata* M280 + PDA (autoclaved cane) - control

T2 = *E. lata* M280 + PDA (gamma-irradiated cane) - control

T3 = *E. lata* M280 + *T. harzianum* strain 1 (autoclaved cane)

T4 = *E. lata* M280 + *T. harzianum* strain 1 (gamma-irradiated cane)

Figure 4.6. Growth of *E. lata* on autoclaved canes in the presence of *T. harzianum*, based on the method of Munkvold and Marois (1993a). Autoclaved cane segments of cv. Shiraz were inoculated with spores or mycelial plugs of *T. harzianum* and incubated in darkness at 23°C. Mycelial plugs of *E. lata* were introduced 24 h later and the segments were incubated for a further 10 days.



T1 = PDA plug + plug of *E. lata* M280 (control)

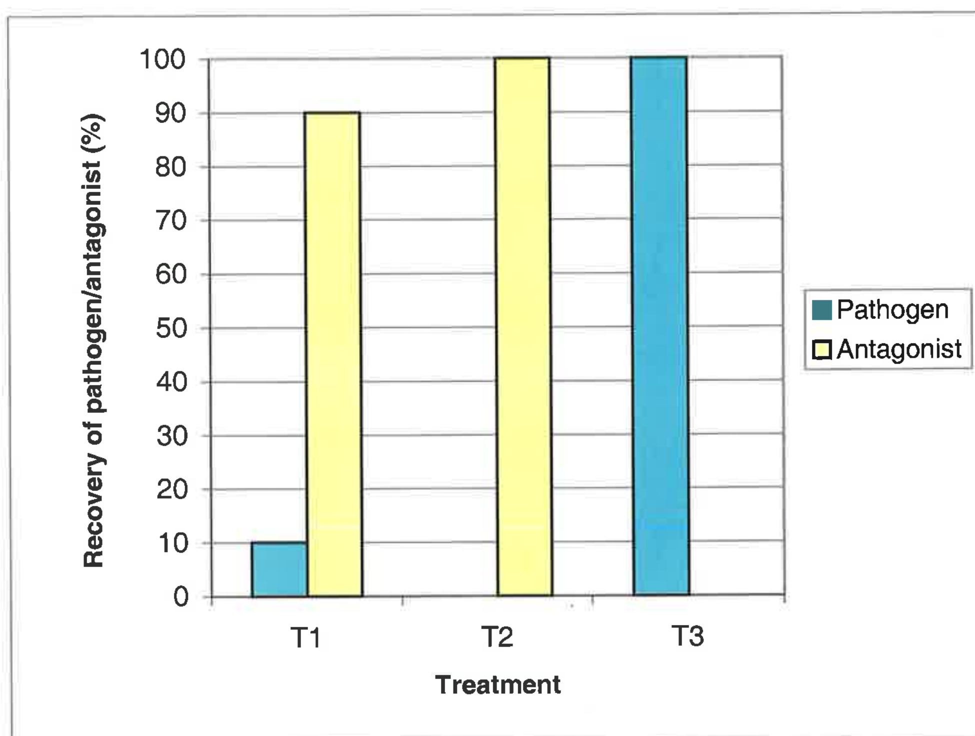
T2 = 10 μl droplet of SDW + plug of *E. lata* M280 (control)

T3 = Plug of *T. harzianum* (strain 1) + plug of *E. lata* M280

T4 = 10 μl droplet of SDW containing 1,500 spores of *T. harzianum* (strain 1) + plug of *E. lata* M280

There were 10 replicates per treatment combination.

Figure 4.7. Colonisation of autoclaved canes by *E. lata* in the presence of spores of *T. harzianum*. Autoclaved cane segments of cv. Shiraz were co-inoculated with a 25 μl droplet containing 1,000 spores each of *E. lata* and *T. harzianum* simultaneously and incubated at 23°C in darkness. Re-isolation of pathogen and antagonist was carried out 4 weeks later.



T1 = *E. lata* ascospores + *T. harzianum* (strain 1) spores

T2 = *T. harzianum* (strain 1) spores alone (control)

T3 = *E. lata* ascospores alone (control)

There were 10 replicates per treatment combination.

4.3.3. Microscopy

The mycelium of *E. lata* was similar in dimensions and branching patterns to that of *T. harzianum*, hence distinguishing the mycelia of the two species in co-inoculated canes using microscopy was not possible. Both the pathogen and the antagonist were observed to grow not only in the vessels but also in the pith parenchyma cells of both the gamma-irradiated canes and the canes grown in rockwool pieces. No hyphae were observed in the un-inoculated canes, although structures resembling rod-shaped bacteria were observed occasionally, predominantly in the vessels.

4.3.3.1. Interaction in gamma-irradiated wood

Turgid and healthy hyphae were observed in the cane pieces that had been inoculated with M280 alone (Figure 4.8 A) or *T. harzianum* alone (Figure 4.8 B). SEM of co-inoculated segments showed hyphae that were flaccid and collapsed (Figure 4.8 C). Similar observations were made for all three replicates.

4.3.3.2. Interaction in cuttings grown in rockwool pieces in the laboratory

The controls, which received *E. lata* alone or *T. harzianum* alone, showed healthy, turgid hyphae. Loss of turgor and abnormal swelling of hyphae were observed in cuttings co-inoculated with mycelial plugs of *E. lata* M280 and 2 days later with spores of *T. harzianum* (Figures 4.9 A and 4.9 B). Hyphae were observed to have lost turgidity and to have shrivelled in the cuttings co-inoculated with mycelial plugs of both pathogen and antagonist (Figure 4.9 C). Abnormal swelling and collapse of hyphae were observed in the cuttings co-inoculated with spores of the pathogen and antagonist (Figure 4.9 D, 4.9

E and 4.9 F). Furthermore, parallel growth and winding or coiling of hyphae were also observed in this material. This may indicate early stages of parasitism.

4.3.3.3. Interactions in simultaneously co-inoculated cuttings grown in rockwool pieces in the laboratory

Spores of *T. harzianum* and *E. lata* were observed to have germinated and produced mycelia in the controls 22 h after inoculation and interacting mycelia were first observed 7 days after inoculation. There were no obvious signs of interactions in cuttings harvested 30 min., 6 h and 22 h after inoculation. Healthy and turgid hyphae were observed at all times of harvest in cuttings that were inoculated with *T. harzianum* or *E. lata* alone (Figures 4.10 A and 4.10 B). In the co-inoculated cuttings, abnormal swelling and collapse of hyphae were generally observed in the cuttings harvested after 7, 14 and 21 days (Figures 4.10 C, 4.10 D and 4.10 F). Also, parallel growth and looping or coiling of hyphae were observed in the cuttings (Figures 4.10 E and 4.10 F). This may be an indication of early stages of parasitic interactions.

Figures 4.8A. - 4.8C. SEM of gamma-irradiated and inoculated cane segments. Pink arrows indicate healthy hyphae of *E. lata* or *T. harzianum*. **Figure 4.8A.** Turgid hyphae of *E. lata* in a xylem vessel adjacent to pith parenchyma in cane segment inoculated with *E. lata* alone. **Figure 4.8B.** Turgid hyphae of *T. harzianum* growing through a pit of a xylem vessel in cane segment inoculated with *T. harzianum* alone. **Figure 4.8C.** Flaccid hyphae in co-inoculated cane segments.

Figures 4.9A - 4.9F. SEM of cuttings inoculated first with *E. lata* and 2 days later with *T. harzianum*. Pink arrows indicate healthy hyphae of *E. lata* or *T. harzianum*. **Figure 4.9A.** Flaccid hyphae in cutting co-inoculated with mycelial plugs. **Figure 4.9B.** Abnormal swelling of hyphae in cutting co-inoculated with mycelial plugs. **Figure 4.9C.** Shrivelled hypha in cutting co-inoculated with mycelial plugs. **Figure 4.9D.** Abnormal swelling of hyphae in cutting co-inoculated with spores. **Figure 4.9E. - 4.9F.** Loss of turgidity and parallel growth of hyphae in cutting co-inoculated with spores.

Figures 4.10A. - 4.10F. SEM of cuttings simultaneously inoculated with spores of *E. lata* and *T. harzianum*. Pink arrows indicate healthy hyphae. **Figure 4.10A.** Turgid hyphae in cutting treated with *E. lata* alone. **Figure 4.10B.** Healthy hyphae of *T. harzianum* in pith parenchyma in cutting treated with *T. harzianum* alone. **Figure 4.10C.** Hyphae showing coiling, abnormal swelling and collapse in co-inoculated cutting. **Figure 4.10D.** Rupture and collapse of hyphae in co-inoculated cutting. **Figure 4.10E.** Parallel growth of hyphae in co-inoculated cutting. **Figure 4.10F.** Coiling and abnormal swelling of hyphae in co-inoculated cutting.

Figure 4.8A.

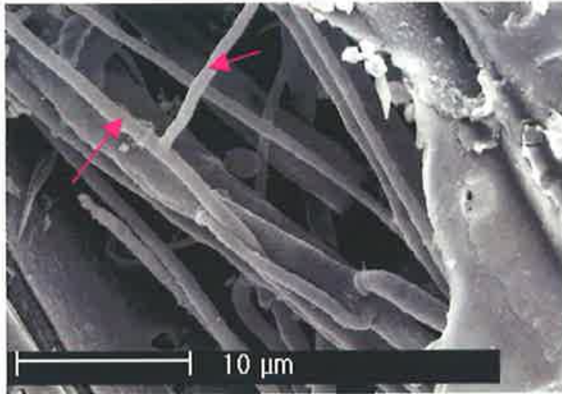


Figure 4.8B.

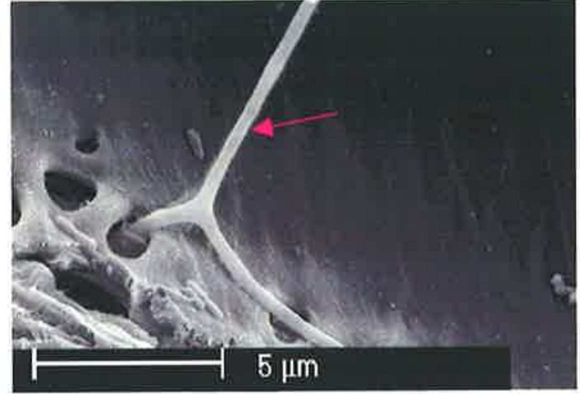


Figure 4.8C.

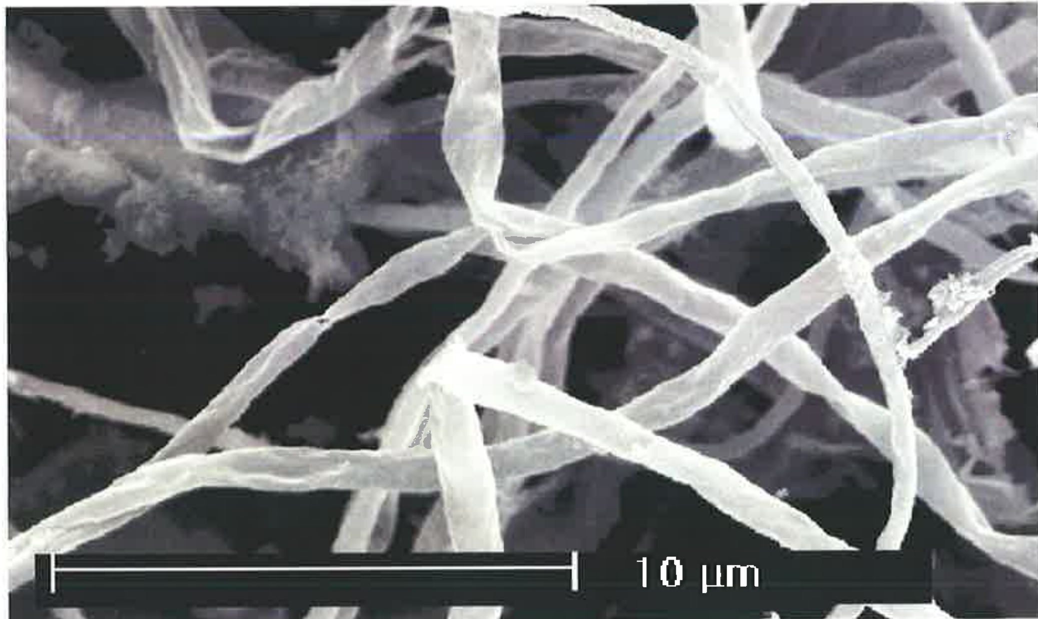


Figure 4.9A.

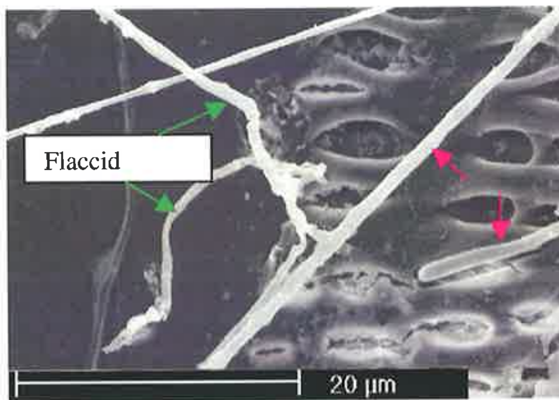


Figure 4.9B.

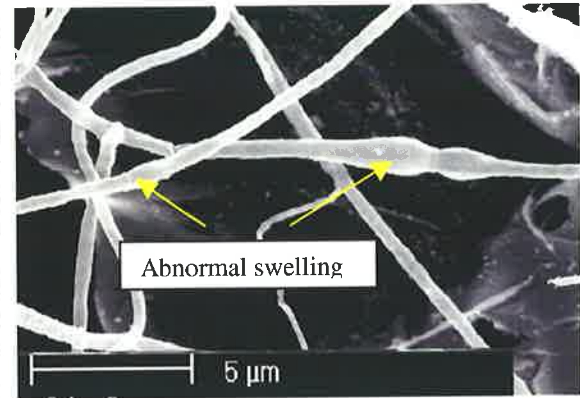


Figure 4.9C.

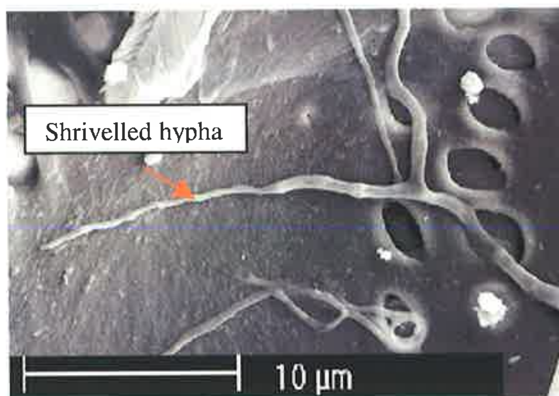


Figure 4.9D.

Figure 4.9E.

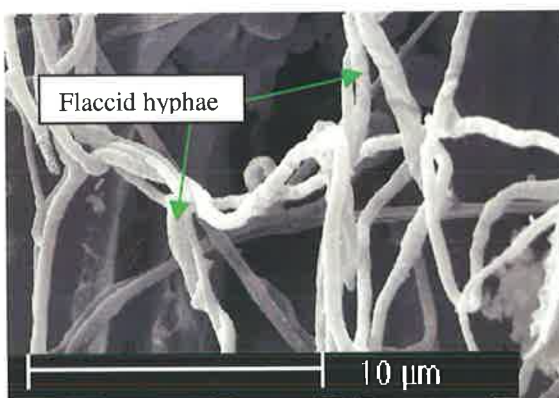


Figure 4.9F.

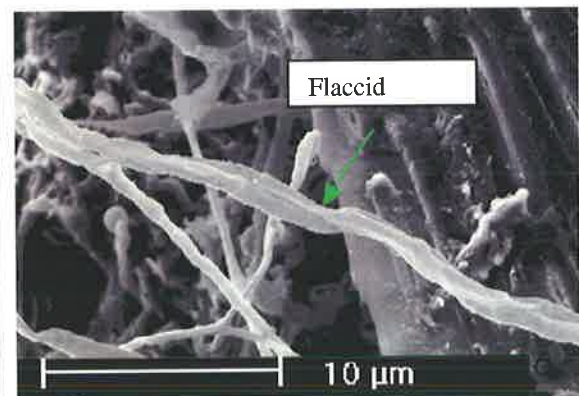


Figure 4.10A.

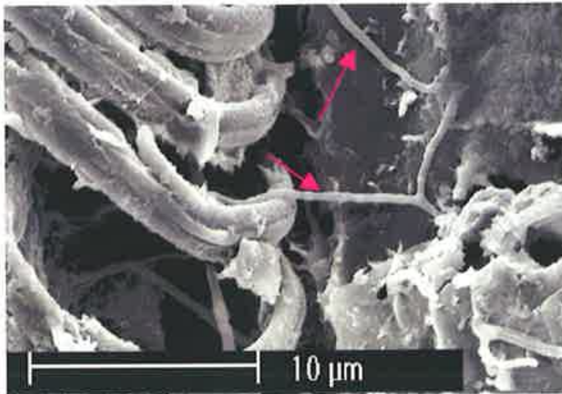


Figure 4.10B.

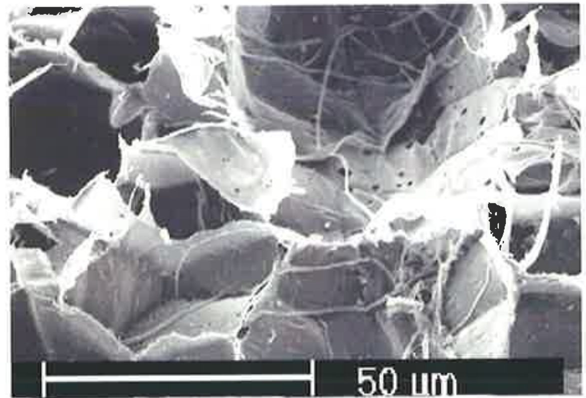


Figure 4.10C.

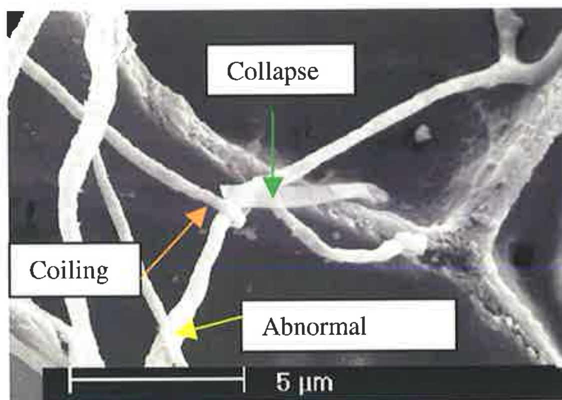


Figure 4.10D.

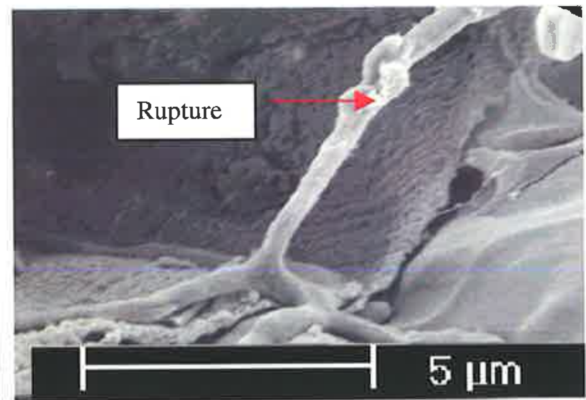


Figure 4.10E.

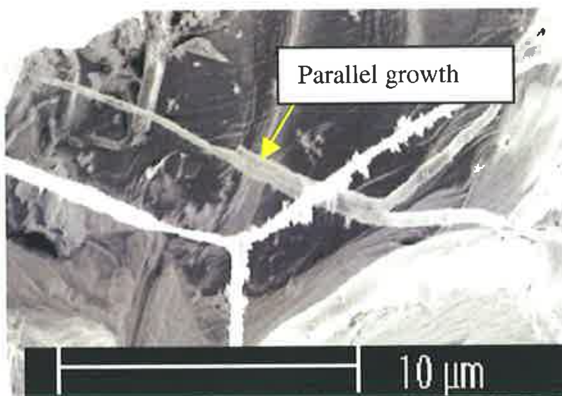
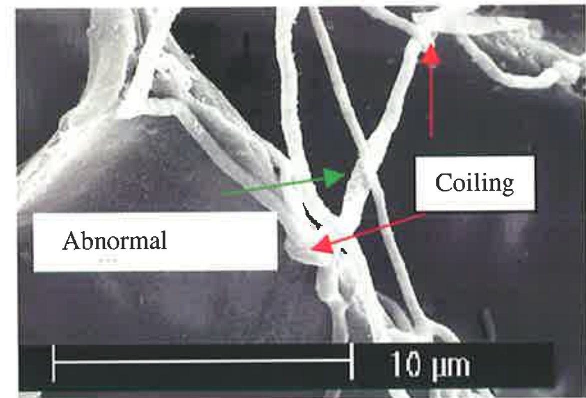


Figure 4.10F.



4.2. Discussion

Mycelial growth of *E. lata* on PDA was inhibited by antibiosis by all three strains of *T. harzianum* but there was no evidence of mycoparasitism when interaction zones of dual cultures were examined using light microscopy. There are many reports of inhibition of plant pathogenic fungi by antibiotic compounds produced by *Trichoderma* spp. (Fravel, 1988; Ghisalberti *et al.*, 1990; Ghisalberti and Sivasithamparam, 1991). Volatile metabolites produced by *Trichoderma* spp. have been shown to inhibit various fungi (see section 2.4.3.4). The three strains used here produced a coconut odour, previously characterized as 6-n-pentyl-2H-pyran-2-one (Claydon *et al.*, 1987), and shown to inhibit mycelial growth of *Ophiostoma ulmi*, *Botrytis cinerea* (Merlier *et al.*, 1985) and *R. solani* (Dennis and Webster, 1971a). Other volatile antibiotics may include isocyanide compounds (Ghisalberti and Sivasithamparam, 1991). Chambers and Scott (1995) reported inhibition of *P. cinnamomi* and *P. citricola* by an odourless, volatile antibiotic that was produced by *T. pseudokoningii*, and partial suppression of growth of *S. rolfsii* was caused by volatile inhibitors produced by *T. virens* (Maiti *et al.*, 1991). Volatile antibiotics alone produced by *Trichoderma* spp. were thought to be responsible for inhibition of the wood decay fungus *Lentinus lepideus* (Bruce *et al.*, 1984). The present study also suggested that the volatile metabolites produced by the three strains of *T. harzianum* might be sufficient to inhibit *E. lata*. However, further testing with more isolates of *E. lata* would be necessary before a conclusion is reached.

The volatile metabolites produced by the three strains of *T. harzianum* inhibited mycelial growth of the two isolates of *E. lata* (Figure 4.1) and had a fungistatic effect on both. Also, volatile metabolites prevented germination of ascospores of *E. lata* (Table

4.1). However, incubation for more than 2 days to observe germination was not possible since the bases of plates inoculated with ascospores and inverted over the bases containing *T. harzianum* were contaminated after 2 days. Hence, whether or not these volatile metabolites had a fungicidal effect on the ascospores of *E. lata* is unknown.

In the experiments designed to assess production of non-volatile metabolites, it was assumed that the inhibition of growth of mycelium or of germination of ascospores of *E. lata* was caused by metabolites of *T. harzianum*, which had diffused through the cellophane membrane into the PDA. Nutrient depletion by the growth of *T. harzianum* was considered unlikely to account for the inhibition of *E. lata* since PDA is rich in nutrients and incubation was only for 2 days (Dennis and Webster, 1971a). The cellophane overlay method has also been used to demonstrate antagonism by non-volatile metabolites produced by *Trichoderma* spp. on other fungi such as *F. annosus, Fusarium oxysporum* and *Phytophthora* spp. (Dennis and Webster, 1971a; Chambers, 1993). Several authors have used non-volatile antibiotics in culture filtrates to demonstrate inhibition by antibiosis without the possibly confounding effects of nutrient depletion. Sivan *et al.* (1984) showed inhibition of growth of *Pythium aphanidermatum* by culture filtrate of *T. harzianum*. Similarly, Chambers and Scott (1995) demonstrated the ability of culture filtrates of *T. hamatum*, *T. pseudokoningii* and *T. virens* to inhibit *P. cinnamomi* and *P. citricola*. However, the culture filtrate method was not used in the present study to demonstrate antibiosis. Carter and Price (1974) demonstrated in a dual culture experiment that non-volatile antibiotics produced by 6-10 day-old *F. lateritium* cultures on Czapek-dox minerals plus yeast extract medium (5% nutrient strength) significantly inhibited germination and mycelial growth of *E. lata*. Their findings further

suggest that depletion of nutrients in the culture medium was unlikely to contribute towards inhibition of the pathogen.

Schirmböck *et al.* (1994) suggested that the group of peptaibols produced by *T. harzianum* was responsible for the antagonistic activity against *B. cinerea* and *Fusarium oxysporum* f. sp. *phaseoli*. Reduction in radial growth of *S. rolfsii* has been attributed to the trichorzianines produced by *T. harzianum* (Correa *et al.* 1995). In the present study, identification of the anti-microbial compounds produced by the three strains of *T. harzianum* was not attempted, and this warrants investigation as part of the evaluation of the potential of *T. harzianum* to control *E. lata*.

The non-volatile metabolites produced by the three strains had a fungistatic effect on some isolates of *E. lata* and a fungicidal effect on others. Also, the degree of inhibition by the three strains of *T. harzianum* varied with the different isolates of *E. lata* (Figures 4.2 and 4.3). This observation suggested that the isolates of *E. lata* may differ in sensitivity to the diffusible metabolites produced by the three strains of *T. harzianum*. This is in agreement with previous work, which demonstrated that isolates of a pathogen may differ in sensitivity towards antagonists (Berg and Ballen, 1994; Mazzola *et al.*, 1995). This feature in *E. lata* is not surprising given the high genetic diversity of this pathogen (Péros *et al.*, 1997; Pèros and Berger, 1999; Péros *et al.*, 1999). Gibbs (1967) showed that *F. annosus* isolates from different geographical locations varied in their sensitivity to antibiotics produced by *Trichoderma* spp. Schmidt *et al.* (2001a) observed that a strain of *Erwinia herbicola* inhibited mycelial growth of the five isolates of *E. lata* mainly by the hydrolases such as chitinase, protease and cellulase they produced on grapevine wood while a sixth isolate was only weakly inhibited by these metabolites.

The difference in the degree of inhibition by the three strains of *T. harzianum* observed in the present study suggests that the three strains may have produced different antibiotics or enzymes (Kullnig *et al.*, 2000) or the same metabolites at varying concentrations. Research has shown that strains belonging to the same species group can differ physiologically in their production of metabolites (Moubasher, 1963; Dennis and Webster, 1971a). Mercer and Kirk (1984a) demonstrated variation in the antagonistic activity of three isolates of *T. viride* towards *Chondrostereum purpureum*, in that, of the three isolates of *T. viride* tested, one strongly inhibited the pathogen, one slightly inhibited and the other one stimulated the mycelial growth of the pathogen.

Ascospores did not germinate in the presence of diffusible metabolites produced by strains 2 and 3 of *T. harzianum* and less than 5% of spores germinated in the presence of metabolites produced by strain 1. In the controls, germination was slightly more than 90% (Figure 4.4). Carter and Price (1974) demonstrated inhibition of germination of ascospores of *E. lata* by non-volatile metabolite(s) produced by *F. lateritium*. They showed that the degree of inhibition by the non-volatile antibiotics produced by 6-day-old cultures of *F. lateritium* varied from 100 to 70% in the absence of nutrients, whereas up to 96% of untreated *E. lata* ascospores germinated after 24 h of incubation. However, the inhibition was not always permanent, as incubation over 24 h led to an increase in the percentage of germination of ascospores of *E. lata*. In a separate time-course experiment they also showed that inhibition of germination of ascospores of *E. lata* increased with the age of *F. lateritium* colony, over 6 successive days. They observed that the germination of ascospores decreased rapidly during the first 2 days and reached 1% at 6 days. Ferreira *et al.* (1991), likewise, observed reduction in ascospore germination in

dual cultures with *Bacillus subtilis*. Similarly, Pachenari and Dix (1980) found that low molecular weight substances from *Gliocladium roseum* inhibited the germination of conidia and subsequent mycelial growth of *Botrytis allii*.

In the experiment based on the method of Munkvold and Marois (1993a), complete inhibition of growth and establishment of *E. lata* mycelia was observed when canes were first inoculated with spores or mycelial plugs of the antagonist (Figure 4.6). Although the *E. lata* isolate used was known to be virulent, it was unable to establish in any of the replicates in the presence of the antagonist. In this experiment, *E. lata* was introduced 24 h after the antagonist. This time interval may have been sufficient for *T. harzianum* to colonise the canes and have a competitive advantage over the pathogen, which was introduced in the form of mycelial plugs. Conidia of *T. harzianum* germinate and initiate mycelial extension in 14 to 18 h at optimal temperature and nutrient conditions (Lifshitz *et al*., 1986; Hjeljord *et al*., 2000). Munkvold and Marois (1993a) reported reduction in infection of 80% or more by ascospores of *E. lata* when applied to autoclaved cane segments embedded upright in water agar pre-inoculated 48 h previously with conidia of *F. lateritium* and *Cladosporium herbarum*. They also reported that 1% of the 348 fungal isolates they had screened prevented germination of ascospores of *E. lata* in wood.

E. lata was not re-isolated from autoclaved cane segments that were inoculated simultaneously with equal concentrations of spores of the antagonist and the pathogen (Figure 4.7). While the reasons for this are not known, it is possible that conidia of *T. harzianum* spores inhibited further development of the germlings of *E. lata* that may have germinated ahead of the conidia. At the optimum temperature of 20-25°C, freshly discharged ascospores were shown to germinate on agar in 11-12 h (Carter, 1991). The

failure of *E. lata* to colonise the canes in this particular experiment could be attributed to the depletion in O_2 and increase in CO_2 levels in the microenvironment since the plates were sealed with Parafilm®, which is likely to have reduced gaseous diffusion. Increase in respiration during germination of conidia and ascospores has been previously reported (Martin and Nicolas, 1970; Rosen *et al.*, 1974).

An obvious criticism of these experiments involving wood is that growth conditions for *T. harzianum* and *E. lata* were optimised by maintaining high humidity and moisture content of the wood and by eliminating competition by the indigenous microflora. Munkvold and Marois (1994) reported the microflora on fresh grapevine pruning wounds to be as low as $10^2 – 10^3$ cfu initially, but this increased to 10^6 within 3-28 days. Furthermore, since the wood tissues were killed by sterilisation, the natural resistance reactions such as accumulation of lignin, suberin and phenolics, and formation of tyloses would not have taken place (Schmidt *et al.*, 2001a). Interactions between pathogen and antagonist may differ somewhat depending on the experimental conditions. For example, Mercer and Kirk (1984a) showed *T. viride* and *T. koningii* to be equally effective in controlling wood decay fungi, such as *Stereum hirsutum*, *Ganoderma applanatum* and *C. purpureum*, when applied as mycelial plugs to autoclaved beech wood strips placed on 2% malt agar and on beech wood strips placed on glass beads in Petri dishes containing distilled water. However, they found other antagonists, such as *F. lateritium* and *Cryptosporiopsis fasiculata*, to be less effective against these pathogens on wood than they had been on agar alone. Reduced antagonistic activity on autoclaved wood has also been reported by others (Toole, 1971; Pratt, 1982). Nevertheless, tests on sterilised wood, generally autoclaved, have proved valuable in the selection of biological agents

for the control of wood pathogens (Mercer and Kirk, 1984b; Ferreira *et al.*, 1990; Munkvold and Marois, 1993a; Hutchinson *et al.*, 1994; Schmidt *et al.*, 2001a).

Although *T. harzianum* is well known for its mycoparasitic activity, the SEM observations suggested that inhibition on gamma-irradiated canes and cuttings grown in rockwool in the laboratory was primarily due to antibiosis (Chet and Baker, 1981; Chet *et al.*, 1981; Elad *et al.*, 1983c). Abnormal swelling, collapse and shrivelling of hyphae were frequently observed in the gamma-irradiated and living co-inoculated canes, which may be attributed to antibiosis. Lifshitz *et al.* (1986) reported abnormal hyphal swelling in *Pythium* sp. that was grown in dual culture plates with *T. harzianum* in water agar medium before mycoparasitism occurred.

Parallel growth and coiling were observed occasionally in the co-inoculated cuttings. These features may indicate early stages of mycoparasitic activity of *Trichoderma* spp., as reported by many researchers (Weindling, 1932; Dennis and Webster, 1971c; Wells *et al.*, 1972; Hennis and Chet, 1975; Tronsmo and Dennis, 1978; Harman *et al.*, 1980; Harman *et al.*, 1981; Elad *et al.*, 1983b; Elad *et al.*, 1983c; Trutmann and Keane, 1990; Benhamou and Chet, 1993; Chambers and Scott, 1995; Benhamou and Chet, 1997). The lytic enzymes chitinase, glucanase and proteases have been reported to be responsible for degradation of fungal cells by mycoparasitic *T. harzianum* (Elad *et al.*, 1982; Chérif and Benhamou, 1990; Benhamou and Chet, 1993; Harman *et al.*, 1993). Some authors have demonstrated the synergistic effect on antifungal activity by antibiotics and the lytic enymes involved in parasitism. Kullnig *et al.* (2000) showed that enzyme diffusion from *Trichoderma atroviride* was necessary before the antagonist could parasitise *R. solani*. Di Pietro *et al.* (1993) reported that an endochitinase and gliotoxin isolated from *T.*

virens did not have a significant effect on germination of conidia of *B.cinerea* when either was applied alone, whereas, when the antibiotic and the enzyme were applied in combination there was a 95% inhibition of germination. A similar effect was observed on *B. cinerea* conidia due to trichorazianines and the lytic enzymes endochitinase, chitobiosidase and glucanase produced by *T. harzianum* (Schirmböck *et al.*, 1994). Lorito *et al.* (1996) used this concept of synergism to obtain high levels of inhibition of *B. cinerea* and *Fusarium oxysporum* using seven different antibiotics and two fungicides in all combinations with eight enzymes from fungi, bacteria or plants. However, the relationship between antibiotics and lytic enzymes of *T. harzianum* and their synergistic effects on *E. lata* was not investigated in the present study. Further investigation of this aspect may be valuable in future studies.

Although coiling and winding were observed in cane segments and cuttings using SEM, they were not detected in the *in vitro* study of dual cultures on PDA using light microscopy (sections 4.3.1.2; 4.3.3.1; 4.3.3.2 and 4.3.3.3). However, parasitism of *E. lata* by *F. lateritium* on agar medium has been reported by Vajna (1986). Nutrient stress is thought to be necessary for the expression of some cell wall degrading enzymes involved in mycoparasitism by *Trichoderma* spp. (Lorito, 1998; Mach *et al.*, 1999). The conditions in wood segments in this case may have been conducive for parasitic interactions between *T. harzianum* and *E. lata* compared to the nutrient-rich PDA medium. Furthermore, increased antibiotic activity has been reported to impede parasitic interactions between UV-induced mutants of *T. harzianum* and *P. ultimum*, *R. solani* and *F. oxysporum* (Graeme-Cook and Faull, 1991). Also, Howell and Stipanovic (1995) showed that gliotoxin-deficient mutants of *T. viride* which had lost antibiotic activity

against *R. solani* were as efficient as the parental strains in controlling disease of cotton seedlings induced by *R. solani*, obviously by a mechanism other than antibiosis. Hence, if strain 1 of *T. harzianum* was a "high" antibiotic producing strain, it may not readily have parasitised *E. lata* in the conditions tested. This hypothesis, however, needs further testing.

In summary, inhibition of germination and mycelial growth of *E. lata* on PDA by *T. harzianum* was primarily by antibiosis. SEM suggested that inhibition in grapevine tissue was mainly by antibiosis but that parasitism may have occurred in cuttings. Spores and mycelial inoculum of *T. harzianum* were capable of inhibiting *E. lata* in grapevine cane segments in laboratory conditions when co-inoculated or when the pathogen was introduced before or after the antagonist. The potential of the antibiotics produced by *T. harzianum* to control *E. lata* should be evaluated and tested on a wide range of isolates of *E. lata* in future studies. Also, the relationship between the antibiotic activity of *T. harzianum* and the lytic enzymes involved in mycoparasitism needs to be investigated.

The results of experiments carried out in this chapter indicated that *T. harzianum* has potential in the biocontrol of *E. lata* on grapevines.

CHAPTER 5. ANTAGONISM OF *E. LATA IN PLANTA* AND COLONISATION OF CANES BY PATHOGEN AND ANTAGONIST

5.1. Introduction

In planta tests in glasshouse conditions are considered by most researchers to be a prerequisite for field experiments. If the candidate organism is unable to control the pathogen or the disease in the glasshouse, it is unlikely to be able to do so in the variable environmental conditions in the field.

The ability of various biological agents to prevent or reduce infection of wounds by fungal pathogens has been evaluated in the glasshouse. Spraying of roses grown in pots, soon after pruning, with a spore suspension of the saprophyte *Ulocladium atrum* reduced infection by *B. cinerea*, by reducing sporulation of the pathogen occurring naturally on senescing plant material in the glasshouse (Kohl and Gerlagh, 1999). Efficacy of stem wound applications of *U. atrum* against infection by *B. cinerea* of tomatoes grown in the glasshouse was shown to be equivalent to the control achieved by treatment with fungicides (Fruit *et al.*, 1999). Wound applications of *T. harzianum* and other fungi such as *Aureobasidium pullulans* and *Cryptococcus albidus* reduced the incidence of stem infection of cucumber plants by *B. cinerea* in the glasshouse (Dik *et al.*, 1999). Non-pathogenic strains of *Fusarium* spp. protected wounds on tomatoes against *B. cinerea* in the glasshouse (Decognet *et al.*, 1999; Trottin *et al.*, 2001).

Wound protection using biological agents has been successful in many tree species. Treatment of pine stumps with *Peniophora gigantea* to protect against infection by

Fomes annosus is widely practised in Britain (Rishbeth, 1963). Application of *P. gigantea* and *Polysporus adustus* to spruce stumps has also shown potential to protect against infection by *Stereum sanguinolentum* (Rishbeth, 1973). Hall *et al.* (1986) demonstrated that colonisation of stems of seedlings of silver and Norway maples by *V. dahliae*, the causal agent of wilt, was reduced when stem wounds were inoculated with isolates of *Bacillus subtilis* prior to the introduction of the pathogen.

Boirie and Pons (1984) showed that the product, Phior-P™, based on *T. harzianum*, when daubed on wounds or sprayed at the time of pruning prevented infection of fruit trees by *C. purpureum*. However, Spiers and Brewster (1997) did not find treatment of wounds on willow and peach trees with *T. viride* to prevent infection by *C. purpureum*.

Infection of pruning wounds on grapevines and apricots by *E. lata* has been prevented by the introduction of various fungi and bacteria (Carter and Price, 1974; Ferreira *et al.*, 1991; Munkvold and Marois, 1993a). Carter and Price (1974) showed that inoculation of freshly pruned sapwood of apricots with *F. lateritium* gave significant protection against infection by *E. lata.* They also reported that *F. lateritiun* was restricted mainly to the sapwood within 2 cm of the pruned surface 6 months after inoculation. Treatment of grapevine pruning wounds with *B. subtilis* significantly reduced infection by *E. lata* when the pathogen was introduced to the wounds 4 h after the antagonist (Ferreira *et al.*, 1991). Munkvold and Marois (1993a) demonstrated biological control of *E. lata* on grapevines by treating pruning wounds with *F. lateritium* and *Cladosporium herbarum*, 2 or 14 days prior to the introduction of the pathogen. Furthermore, Munkvold and Marois (1993a) found *T. viride* to be moderately effective in reducing infection of grapevine pruning wounds by *E. lata* and inconsistent in comparison with *F. lateritium*

and *C. herbarum*. However, apart from these reports of biological control of *E. lata*, information is lacking on the interactions between *E. lata* and fungal antagonists. Further information might lead to optimising biological protection of pruning wounds on grapevines, which would facilitate the development of commercial biological control products.

The objectives of the experiments described in this section were to test antagonism of *T. harzianum* against *E. lata in planta*, to evaluate different methods of introduction of the antagonist into grapevine cuttings and the extent of subsequent colonisation. *F. lateritium*, which is a known antagonist of *E. lata*, was used for the sake of comparison in some experiments (Carter and Price, 1974; Carter, 1983). The technique used to inoculate the canes under the upper bud in some of the experiments was adapted from the method used by Péros and Berger (1994) to test the pathogenicity of *E. lata* isolates on grapevine canes.

5.2. Materials and methods

5.2.1. Experiment 5.1. Colonisation of cuttings by antagonists

The aim of this experiment was to study colonisation of cane cuttings by the antagonists when introduced as mycelial plugs or spore suspensions on pruning wounds made above the upper bud or into holes drilled under the upper bud. Cane cuttings of Shiraz were allowed to stand in tap water overnight after the third basal bud was removed (see section 3.1; Table 3.1) then inoculated as follows.

Pruning wounds were made on 96 cuttings, 4 cm above the upper bud and the cut surface was inoculated ("**top inoculation**") with one of the following treatments:

Treatment 1 = *F. lateritium* spores (40 μl droplet)

Treatment 2 = mycelial plugs (5 mm diam.) of *F. lateritium* on CDA

Treatment 3 = *T. harzianum* (strain 1) spores (40 μl)

Treatment 4 = mycelial plugs (5 mm diam.) of *T. harzianum* (strain 1) on PDA

Treatment 5 = SDW (40 μl)

Treatment 6 = sterile PDA plug (5 mm diam.)

The concentration of the spore suspensions of both *F. lateritium* and *T. harzianum* used in this experiment was 10^9 spores/ml (see section 3.3.1).

Another 96 cuttings were inoculated via holes (5 mm diam.) drilled 3 cm below the upper bud, the treatments listed above were administered ("**middle inoculation**") into the holes and the wound covered with Parafilm®.

The cuttings were harvested at two times; there were eight replicates per treatment for each of the "top" and "middle inoculated" positions at each harvest. The experiment was a split plot design with no blocks. Twelve canes were arranged in each of 16 trays (section 3.1). Within a tray, each cane received one of the 12 combinations of treatment (T1 – T6) and position of inoculation. The cuttings were maintained in the glasshouse as described in section 3.1. Half the replicates were harvested 10 weeks after inoculation (96 canes), and re-isolation of the antagonists was carried out (see section 3.4.2) from the discoloured tissue (i.e. 3-5 mm below the wound) as well as the healthy tissue (i.e. up to 2 cm below the point of inoculation after removal of discoloured tissue). Transverse sections were also cut by hand using double-edged Gillette® Silver Blue razor blades,

from three replicates of each treatment combination, just beyond the margin of the discoloured tissues. The sections were mounted in cotton blue in lactophenol and observed with the light microscope (Leitz Orthoplan 871288) for the presence of spores or mycelia within the xylem vessels.

The other 96 canes were harvested 20 weeks after inoculation. In this case, re-isolation of the antagonists was from the discoloured tissues at the wound site as well as from wood at 1, 2 and 4 cm below the point of inoculation for the "top" and "middle inoculated" canes (see section 3.4.2). Additionally, re-isolation was carried out at 1, 2 and 4 cm above the point of inoculation for the "middle inoculated" cuttings.

Logistic regression was performed on the results (see section 3.6) for re-isolation of the antagonists from different positions on the canes 10 and 20 weeks after inoculation. The control treatments with SDW and sterile PDA plugs were not included in the analysis since isolation yielded no fungi, confirming that the canes were not contaminated with the antagonist.

5.2.2. Experiment 5.2. Effect of *T. harzianum* on infection of cuttings by *E. lata*

The aim of this experiment was to study the effect of *T. harzianum* on infection from mycelial plugs of *E. lata* in grapevine cuttings when introduced at a distance from the antagonist on the same day. Holes (5 mm diam.) were drilled 1 cm below the upper bud in 60 canes each of cultivars Chardonnay and Shiraz (see section 3.1, Table 3.1). The holes in 30 canes of each cultivar were inoculated with 5 mm diam. plugs of *T. harzianum* (strain 1) while the other 30 canes of each cultivar were inoculated with plugs of sterile PDA of the same dimensions. Holes of 3 mm in diam. were drilled 1 cm below

the upper bud in another 60 canes each of cultivars Chardonnay and Shiraz and the holes in 30 of the canes of each cultivar were inoculated with 40 μl of spore suspension (10^9 spores/ml) of *T. harzianum* strain 1 (see section 3.3.1). The other 30 canes of each cultivar were inoculated with 40 μl of SDW.

On the same day, each cane was pruned 4 cm above the upper bud and a mycelial plug (5 mm diam.) of *E. lata* isolate M280 placed on the cut surface, then covered with Parafilm®. The experiment was set up in a split plot design with no blocks. There were 10 replicates per treatment combination. There were 30 trays, each containing eight canes representing the eight treatment combinations. The canes were harvested 11, 14 and 20 weeks after inoculation. The discoloured portion from top of the cane (3-5 mm) was discarded and re-isolation of *E. lata* from 1 cm segments below the point of inoculations was carried out as described in section 3.4.1.

5.2.3. Experiment 5.3. Effect of antagonists on infection of cuttings by *E. lata*

The aim of this experiment was to study the effect of *T. harzianum* and *F. lateritium* on infection by *E. lata in planta* when introduced at a distance from the antagonist at three different time intervals. Mycelial plugs (5 mm diam.) of *T. harzianum* (strain 1) or *F. lateritium* were inserted into holes, 5 mm diam., drilled 2.5-3 cm below the upper buds of 64 cuttings of cultivar Chardonnay (see section 3. 1, Table 3.1). Plugs of sterile PDA of the same dimensions were applied to 32 canes as controls. A further 32 canes were treated with Benlate®, instead of inoculation with *T. harzianum* or *F. lateritium*.

All cuttings were pruned 4 cm above the upper bud on Day 0 and those designated fungicide-treated controls were immediately painted with Benlate® (25 g/L). The pruning

wounds on all cuttings were inoculated with plugs of *E. lata* isolate M280 or sterile PDA (5 mm diam.) 1 and 7 days after pruning and treatment with the antagonists or Benlate®. There were eight replicates for each of the 12 treatment combinations. The inoculum of the pathogen and antagonists was covered with Parafilm®. The cuttings were maintained in the glasshouse as described in section 3.1. The experiment was a split plot design with no blocks. Twelve canes were arranged in each of 16 trays. Within a tray, each cane received one of the 12 treatment combinations. The canes were harvested 11 weeks and 8 months after the introduction of antagonists or the treatment with Benlate®. The discoloured wounded portion (3-5 mm) was discarded and 1 cm segments from the top of the canes were excised for re-isolation of *E. lata* as described in section 3.4.1.

5.2.4. Experiment 5.4. Protection of pruning wounds from infection by *E. lata*

This experiment was designed to investigate the ability of *T. harzianum* strain 1, applied as a spore suspension in SDW and in Trichoseal® base, to prevent infection of pruning wounds by *E. lata*. Cane cuttings of cultivar Shiraz were pruned 4 cm above the upper bud and the following treatments were administered to the wounds using 25 mm flat varnish brushes (see section 3.1; Table 3.1). Separate brushes were used for each treatment to avoid cross-contamination.

Treatment 1 (T1) = *T. harzianum* (strain 1) in Trichoseal® base (100 g/L)

Treatment 2 (T2) = *T. harzianum* (strain 1) spore suspension (10^9 spores/ml SDW)

Treatment 3 (T3) = gamma-irradiated *T. harzianum* (strain1) in Trichoseal® base (100g/L)

Treatment 4 (T4) = SDW

Plugs (5 mm diam.) of *E. lata* isolate M280 were introduced to the wounds 0, 2 and 7 days after the above treatments were administered, and covered with Parafilm®. Each treatment combination, which was randomly assigned to the canes in the glasshouse in a 12 x 8 (row x column) design, was replicated eight times. The treatment combinations were applied in a factorial arrangement with four types of treatment with the antagonist or SDW control and three different times for the introduction of the pathogen. The canes were harvested 12 weeks after the application of treatments T1- T4, and re-isolation of *E. lata* was performed (see section 3.4.1) from the wood 2 cm below the point of inoculation after the discoloured wound (3-5 mm) was discarded.

5.2.5. Experiment 5.5. Protection of pruning wounds with Trichoseal®

This experiment was similar to Experiment 4, except that two additional treatments were included and *E. lata* isolate M280 was applied 2 and 7 days after the antagonists. The additional treatments were (i) Trichoseal® and (ii) gamma-irradiated Trichoseal® as the corresponding control (see Table 3.3). The treatment combinations were applied in a factorial arrangement with six types of treatment with the antagonist and two different times of application of the pathogen. Re-isolation of *E. lata* was carried out as above 12 weeks after the pruning wounds were treated with the antagonist (see section 3.4.1).

This experiment was repeated but with re-isolation of *E. lata* and *T. harzianum* carried out simultaneously (see sections 3.4.1 and 3.4.2) at regular distances along the length of the canes by excising 2 cm segments up to a distance of 10 cm below the point of inoculation. Re-isolation along the whole length of the cane was carried out only for cuttings inoculated on Day 2. The data for the two trials were analysed separately.

Logistic regression was performed for the isolation data 2 cm below the point of inoculation for the repeated trial.

5.2.6. Experiment 5.6. Protection by prior inoculation with antagonist

The aim of this experiment was to investigate the ability of *T. harzianum* strain 1 to protect grapevine cuttings from *E. lata* when inoculated prior to, and at a different position from, the pathogen. Holes (5 mm diam.) were drilled into 30 cuttings of cultivar Chardonnay 1.5 cm below the upper bud. Plugs of *T. harzianum* strain 1 (5 mm diam.) were inserted into the holes in 20 canes while plugs of sterile PDA were inserted into 10 canes. Inoculation sites were covered with Parafilm® (see section 3.1; Table 3.1). The canes were arranged in a 10 x 3 (row x column) design in rockwool pieces in pots as described in section 3.1. After 7 days, 5-mm diam. holes were drilled 1 cm below the upper bud and inoculated with 5 mm diam. plugs of *E. lata* isolate M280 or sterile PDA. Ten of the 20 canes that had been treated with plugs of *T. harzianum* received plugs of *E. lata* while the other 10 canes were treated with plugs of sterile PDA. The 10 canes which were initially treated with PDA plugs were also inoculated with *E. lata*. All treatments were randomly administered to the canes. There were 10 replicates for each of the three treatment combinations. The canes were harvested 12 weeks after the introduction of *T. harzianum*. The canes were cut into 2 cm pieces along the whole length and re-isolation of *E. lata* and *T. harzianum* was carried out simultaneously (see sections 3.4.2 and 3.4.1) to estimate the extent of colonisation of wood by the pathogen and the antagonist.

Logistic regression (see section 3.6) was performed on the results to evaluate the effect of *T. harzianum* on *E. lata* when the pathogen and antagonists were in close proximity to each other but inoculated at different points.

5.3. Results

5.3.1. Experiment 5.1. Extent of colonisation by antagonists

Microscopic examination of transverse sections of canes did not reveal spores or mycelium of the antagonists within the xylem vessels.

Re-isolation of antagonists from the discoloured tissue:

Both antagonistic fungi were re-isolated from the discoloured tissue from the "top" and "middle inoculated" canes, at 10 and 20 weeks. However, *T. harzianum* appeared not to have persisted in, or to have declined in, the discoloured tissue when applied as spores. Neither fungus was re-isolated from the un-inoculated controls (Tables 5.1-5.3).

The logistic regression (see section 3.6) performed for the results for re-isolation of antagonists from the discoloured tissues indicated that there was a significant interaction (P=0) between the treatments and the position of inoculation (i.e. "top" or "middle" inoculation). Also, there was a significant interaction (P=0.03) between the time of harvests (i.e. 10 or 20 weeks) and the position of inoculation. The predicted probabilities of re-isolating antagonists from the discoloured wound tissues at the "top inoculated" canes were high, 1.00 for canes treated with mycelium of *F. lateritium* and mycelium of *T. harzianum*, whereas canes that were treated with spores of *T. harzianum* did not yield

the antagonist (Table 5.4). The predicted probability of re-isolating *F. lateritium* from discoloured tissues of "top inoculated" canes was 0.75. Furthermore, the probabilities of re-isolating the antagonists from the discoloured tissues were similar for the "top" and "middle inoculated" canes, 0.72 and 0.66 respectively, harvested 10 weeks after inoculation (Table 5.5). The antagonists were more likely to be isolated from dead tissues of canes that were "top inoculated" and harvested 20 weeks after inoculation than from "middle inoculated" canes, predicted probabilities being 1.00 and 0.56 respectively.

Re-isolation of antagonists at 1 cm below the point of inoculation:

T. harzianum persisted 1 cm below the point of inoculation at 20 weeks in the cuttings that were "top-inoculated" only when applied as plugs of mycelia, whereas it could not be re-isolated at this distance from cuttings treated with spores (Table 5.2). In the "middle inoculated" canes, however, *T. harzianum* was re-isolated at this distance in more than half the cuttings that were treated with spores whereas it persisted in all the cuttings treated with the plugs of mycelia at 20 weeks (Table 5.3). *F. lateritium* persisted at 1 cm in 60-100% of the "top" and "middle inoculated" cuttings (Tables 5.2 and 5.3). Neither fungus was re-isolated from the un-inoculated controls. The logistic regression analysis (see section 3.6) for re-isolation of antagonists 20 weeks after inoculation indicated that there was no significant interaction between the effects of position of inoculation ("top" and "middle") and the treatments (P=0.06). However, the main effects of treatment (P=0) and position of inoculation (P=0.01) were significant. The probabilities for re-isolating the antagonists at 1 cm below the point of inoculation for the treatments with spores and mycelium of *F. lateritium* and mycelium of *T. harzianum*

were high, being 0.88, 0.94 and 1.00, respectively, compared to those treated with spores of *T. harzianum* which was 0.31 (P=0) (Table 5.6). Also, the probability for re-isolating the antagonist 1 cm below the point of inoculation for the "middle inoculated" canes was significantly higher, at 0.87 (P=0.01), than that for the "top inoculated" canes, 0.69 (Table 5.7).

Table 5.1. Experiment 5.1: Number of canes, of eight, yielding antagonists from the discoloured and living tissues (2 cm below point of inoculation) 10 weeks after application of inoculum to pruning wounds ("top inoculation") or below the upper bud ("middle inoculation").

	"Top inoculation"		**"Middle inoculation"**	
Treatment	**Discoloured tissue**	**Living tissue**	**Discoloured tissue**	**Living tissue**
1. *F. lateritium* spores	7	7	8	8
2. *F. lateritium* myceliun	8	8	8	8
3. *T. harzianum* strain 1 spores	0	8	8	8
4. *T. harzianum* strain 1 mycelium	8	8	8	8
5. SDW	0	0	0	0
6. PDA plug	0	0	0	0

Table 5.2. Experiment 5.1: Number of "top inoculated" canes, of eight, yielding antagonists at various distances below point of inoculation after 20 weeks.

Treatment	Discoloured tissue	1 cm below	2 cm below	4 cm below
1. *F. lateritium* spores	5	6	6	8
2. *F. lateritium* myceliun	8	8	8	8
3. *T. harzianum* strain 1 spores	0	0	0	0
4. *T. harzianum* strain 1 myceliun	8	8	8	7
5. SDW	0	0	0	0
6. PDA plug	0	0	0	0

Table 5.3. Experiment 5.1: Number of "middle inoculated" canes, of eight, yielding antagonists at various distances above and below the point of inoculation after 20 weeks.

Treatment	Discolour -ed tissue	1 cm below	2 cm below	4 cm below	1 cm above	2 cm above	4 cm above
1. *F. lateritium* spores	8	8	8	8	7	8	6
2. *F. lateritium* myceliun	7	7	8	7	8	6	8
3. *T. harzianum* strain 1 spores	2	5	5	8	8	8	8
4. *T. harzianum* strain 1 mycelium	1	8	8	8	8	8	6
5. SDW	0	0	0	0	0	0	0
6. PDA plug	0	0	0	0	0	0	0

Table 5.4. Experiment 5.1: Predicted probabilities of re-isolating antagonists, irrespective of the harvest times, from the discoloured wound tissues of "top" and "middle inoculated" canes when each treatment was applied to eight replicates.

Treatment	Predicted probabilities	
	Top	Middle
1. *F. lateritium* spores (40 μl droplet, 10^9 spores/ml).	0.75	1.00
2. *F. lateritium* mycelial plugs (5 mm diam.).	1.00	0.94
3. *T. harzianum* (strain 1) spores (40 μl droplet, 10^9 spores/ml).	0.00	0.62
4. Treatment 4 = mycelial plugs of *T. harzianum* strain 1 (5 mm diam.).	1.00	0.56

Treatment 5 (40 μl SDW) and Treatment 6 (sterile PDA plug) were not included in the analysis, as no fungi were isolated from the cane tissues.

Table 5.5. Experiment 5.1: Predicted probabilities of re-isolating the antagonists from the discoloured tissues of "top" and "middle inoculated" canes harvested 10 and 20 weeks after inoculation.

Position of inoculation	Predicted probabilities at the two Harvests	
	10 weeks	20 weeks
"Top inoculation"	0.72	1.00
"Middle inoculation"	0.66	0.56

Re-isolation of antagonists at 2 cm below the point of inoculation:

T. harzianum and *F. lateritium* persisted 2 cm below the point of inoculation in almost all of the cuttings at 10 weeks (Table 5.1). However, *T. harzianum* was not recovered at this distance after 20 weeks from the cuttings that were "top inoculated" with spores, while more than 50 % of the cuttings that were "middle inoculated" with the spores yielded *T. harzianum* (Tables 5.2 and 5.3). *F. lateritium* persisted in the majority of the "top" and "middle" inoculated cuttings at 20 weeks, while none of the controls yielded the antagonists (Tables 5.2 and 5.3).

The logistic regression model indicated a significant interaction between the time of harvest (10 or 20 weeks after inoculation) and the treatments (P=0.04). Furthermore, the main effect of the position of inoculation ("top" or "middle inoculation") was significant (P=0). The probabilities for re-isolating the antagonists 2 cm below point of inoculation were high (P=0.04) for canes that were treated with the spores and mycelium of *F. lateritium* and mycelium of *T. harzianum* when the canes were harvested after 10 and 20 weeks (Table 5.8). The probabilities of antagonists existing at 2 cm below point of inoculation were significantly higher (P=0) for the "middle inoculated" canes than for those which were "top inoculated" (Table 5.9).

Table 5.6. Experiment 5.1: Predicted probabilities of re-isolating antagonists from wood that was 1cm below the point of inoculation when canes were harvested 20 weeks after inoculation and each treatment was administered to eight replicates.

Treatment	Probabilities
1. *F. lateritium* spores (40 μl droplet, 10^9 spores/ml).	0.88
2. *F. lateritium* mycelial plugs (5 mm diam.).	0.94
3. *T. harzianum* (strain 1) spores (40 μl droplet, 10^9 spores/ml).	0.31
4. mycelial plugs of *T. harzianum* strain 1 (5 mm diam.)	1.00

Treatment 5 (40 μl SDW) and Treatment 6 (sterile PDA plug) were not included in the analysis, as no fungi were isolated from the cane tissues.

Table 5.7. Experiment 5.1: Predicted probabilities of re-isolating antagonists from 1 cm below the point of inoculation for the "top" and "middle inoculated" canes when these were harvested 20 weeks after inoculation.

"Top inoculation"	"Middle inoculation"
0.69	0.87

Table 5.8. Experiment 5.1: Predicted probabilities of re-isolating the antagonists at 2 cm below the point of inoculation for canes harvested 10 and 20 weeks after inoculation. There were eight replicates per treatment.

Treatment	10 weeks	20 weeks
1. *F. lateritium* spores (40 μl droplet, 10^9 spores/ml).	0.94	0.87
2. *F. lateritium* mycelial plugs (5 mm diam.)	1.00	1.00
3. *T. harzianum* strain 1 spores (40 μl droplet, 10^9 spores/ml).	1.00	0.31
4. Mycelial plugs of *T. harzianum* strain 1 (5 mm diam.)	1.00	1.00

Treatment 5 (40 μl SDW) and Treatment 6 (sterile PDA plug) were not included in the analysis, as no fungi were isolated from the cane tissues.

Table 5.9. Experiment 5.1: Predicted probabilities for re-isolating the antagonists at 2 cm below point of inoculation from canes that had been inoculated at the "top" and "middle positions".

"Top inoculation"	"Middle inoculation"
0.83	0.95

Re-isolation of antagonists from 4 cm below the point of inoculation:

At 20 weeks both the antagonists persisted at 4 cm below the point of inoculation in the majority (> 60 %) of the cuttings (Tables 5.2 and 5.3) but, *T. harzianum* did not persist in the cuttings at this distance when applied as spores. None of the un-inoculated controls yielded the fungus.

The regression model indicated a significant interaction between the position of inoculation and the treatments (P=0). The probability of re-isolating the antagonists from canes treated with spores and mycelium of *F. lateritium* and mycelium of *T. harzianum* was high for canes inoculated at both positions, while the probability of re-isolating *T. harzianum* 4 cm below the point of inoculation in canes that had been inoculated at the top with spores of *T. harzianum* was low (Table 5.10).

Re-isolation of antagonists at 1 cm above point of inoculation:

Both fungi persisted 1 cm above the point of inoculation at 20 weeks in 85-100% of the cuttings (Table 5.3). The logistic regression performed on the results for re-isolation of antagonists indicated there was no significant effect of the treatments (P=0.41). The model predicted a high probability of 0.97 for re-isolating both antagonists at 1 cm above the point of inoculation.

Re-isolation of antagonists at 2 cm above point of inoculation:

Both fungi were re-isolated from 60-100% of the cuttings at 20 weeks (Table 5.3). There were no significant treatment effects (P=0.11) and the probability of re-isolating the antagonists at 2 cm above the point of inoculation was 0.94 for all treatments.

Re-isolation of antagonists at 4 cm above point of inoculation:

Both fungi persisted 4 cm above the point of inoculation at 20 weeks in more than 60% of the cuttings (Table 5.3). There was no significant difference between the four treatments (P=0.11). The probability of re-isolating the antagonists 4 cm above the point of inoculation was 0.89.

Table 5.10. Experiment 5.1: Probabilities for re-isolating antagonists at 4 cm below the point of inoculation from the "top" and "middle inoculated" canes when these were harvested 20 weeks after inoculation. There were eight replicates per treatment.

Treatment	"Top inoculation"	"Middle inoculation"
1. *F. lateritium* spores (40 μl droplet, 10^9 spores/ml).	1.00	1.00
2. *F. lateritium* mycelial plugs (5 mm diam.).	1.00	0.88
3. *T. harzianum* (strain 1) spores (40 μl droplet, 10^9 spores/ml).	0.00	1.00
4. mycelial plugs of *T. harzianum* strain 1 (5 mm diam.).	0.88	1.00

Treatment 5 (40 μl SDW) and Treatment 6 (sterile PDA plug) were not included in the analysis, as no fungi were isolated from the cane tissues.

5.3.2. Experiment 5.2. Protection of cuttings by *T. harzianum*

E. lata was re-isolated from all segments of canes of Chardonnay and Shiraz inoculated with the pathogen plus mycelial plugs or spores of *T. harzianum*, PDA or SDW on the same day, 1 cm below the upper bud, regardless of time of harvest. Statistical analysis, therefore, was not warranted.

5.3.3. Experiment 5.3. Protection of cuttings by antagonists

E. lata was recovered from the majority of the canes (60-100% of the cuttings) treated with the antagonist 1 and 7 days before the pathogen, at both times of harvest (Table 5.11). All cuttings treated with *E. lata* alone also yielded the pathogen at the two harvest times. However, *E. lata* was not isolated from any of the cuttings treated with antagonists alone nor from those treated with *E. lata* and Benlate® and harvested after 11 weeks. *E. lata* was isolated from some of the Benlate®-treated cuttings 8 months after inoculation (Table 5.11).

According to the logistic regression analysis there were no interactions at the 5% level. The main effects of treatment (P=0) and harvest times (P=0.04) were significant and, therefore, were examined separately. The difference between the two times of inoculation of *E. lata* after the antagonists (i.e. Day 1 and Day 7) was not significant (P=0.21). This indicated that application of *T. harzianum* or *F. lateritium* 1 or 7 days before *E. lata* did not prevent colonisation of the canes by the pathogen (Table 5.12). Benlate® was the only treatment that inhibited colonisation by *E. lata*. The probability of re-isolating *E. lata* from the canes 8 months after inoculation was significantly higher (P=0.04) than at 11 weeks (Table 5.13).

Table 5.11. Experiment 5.3: Number of canes, of eight, yielding *E. lata* from 1 cm segments excised from the top of canes after 11 weeks or 8 months. The pathogen was introduced 1 or 7 days after application of the antagonists or Benate®.

	Harvested at 11 weeks		**Harvested at 8 months**	
	Day 1	**Day 7**	**Day 1**	**Day 7**
T. harzianum strain 1+ *E. lata* M280	8	8	8	8
F. lateritium + *E. lata* M280	6	8	8	7
PDA plug + *E. lata* M280	8	8	8	8
Benlate + *E. lata* M280	0	0	1	3
T. harzianum strain 1+ PDA	0	0	0	0
F. lateritium + PDA	0	0	0	0

Table 5.12. Experiment 5.3: The probability of re-isolation of *E. lata* from canes treated with the antagonists (eight replicates per treatment) when harvested 12 weeks after inoculation with the antagonists as estimated by the logistic regression model.

Treatment	**Probability of re-isolation of *E. lata***
T. harzianum strain 1+ *E. lata* M280	1.00[a]
F. lateritium + *E. lata* M280	0.91[a]
PDA plug + *E. lata* M280	1.00[a]
Benlate + *E. lata* M280	0.13[b]

Probabilities with the same letters are not significantly different.

Table 5.13. Experiment 5.3: The probability of canes (eight replicates per treatment) yielding *E. lata* at each time of harvest as estimated by the regression model across all treatments.

11 Weeks	8 months
0.72[c]	0.80[d]

Probabilities with the same letters are not significantly different.

5.3.4. Experiment 5.4. Protection of pruning wounds

E. lata was recovered from all the replicates of the controls inoculated with the pathogen 2 and 7 days after treatment with the antagonist. *E. lata* was recovered in all of the cuttings inoculated with the pathogen on Day 0 except that it was re-isolated from only three of eight cuttings treated with gamma-irradiated *T. harzianum* strain 1 in Trichoseal® base. Also, the pathogen was recovered from one of eight and two of eight cuttings inoculated with *E. lata* 2 days after treatment with *T. harzianum* strain 1 in Trichoseal® base and *T. harzianum* strain 1 spores, respectively, and *vice versa* from cuttings inoculated with *E. lata* 7 days after the antagonist (see Appendix 3).

The logistic regression indicated that there were significant interactions ($P<0.001$) between the effects of treatment and day at the 5% level. *T. harzianum* (strain 1), both in the form of spore suspension in SDW and in the Trichoseal® base, reduced ($P<0.001$) colonisation of the canes by *E. lata* when the pathogen was introduced after 2 or 7 days after the antagonist (Table 5.14). Introducing the pathogen at the two different times (Day 2 and Day 7) did not have a significant impact on infection by *E. lata* (Table 5.14). Colonisation by *E. lata* was not reduced compared to the controls (SDW) when the pathogen and antagonist were applied on the same day. However, the canes treated with

gamma-irradiated *T. harzianum* strain 1 in Trichoseal® showed significant increase in infection by *E. lata* ($P<0.001$), when introduced 2 or 7 days after the antagonist compared to those treated with *T. harzianum* strain 1 in Trichoseal® and inoculated with *E. lata* on the same day.

Table 5.14. Experiment 5.4: Predicted probabilities of infection by *E. lata* of canes as estimated by the logistic regression model. *E. lata* was introduced 0, 2 and 7 days after the antagonist and the canes were harvested 12 weeks later. There were eight replicates per treatment.

	Probabilities that canes are infected by *E. lata*			
Day of inoculation of *E. lata*	T1	T2	T3	T4
0	0.99[a] (0.00)	0.99[c] (0.00)	0.43[e] (0.19)	0.99[g] (0.00)
2	0.13[b] (0.12)	0.38[d] (0.17)	0.99[f] (0.00)	0.99[g] (0.00)
7	0.25[b] (0.15)	0.13[d] (0.12)	0.99[f] (0.00)	0.99[g] (0.00)

Probabilities with the same letters in each column are not significantly different. Standard error values in parentheses.

Treatment 1 (T1) = *T. harzianum* (strain 1) in Trichoseal® base (100 g/L)

Treatment 2 (T2) = *T. harzianum* (strain 1) spore suspension (10^9 spores/ml)

Treatment 3 (T3) = Gamma-irradiated *T. harzianum* (strain 1) in Trichoseal® base (100 g/L) (control)

Treatment 4 (T4) = SDW (control)

5.3.5. Experiment 5.5. Pruning wound treatments

Recovery of *E. lata* was 100% in the controls that were treated with non-viable *T. harzianum* or SDW while infection by *E. lata* was reduced in canes that were treated with *T. harzianum* in both repetitions of the experiment (Table 5.15). Logistic regression was performed for the results of re-isolations of *E. lata* from wood 2 cm below the pruning wound to estimate the efficacy of the preventive treatments on infection by *E. lata*. In the first trial, there was no interaction between the effects of treatment and the day of introduction of *E. lata* ($p=0.836$). The three treatments containing viable *T. harzianum* propagules reduced ($P<0.001$) infection by *E. lata* as shown in Table 5.16, irrespective of the day (Day 2 and Day 7) of introduction of *E. lata* ($P=0.372$) when compared to the control treatments of the first trial.

Analysis of data of the repeat trial of experiment 5 indicated that there were no interactions between the treatment and the day effects ($P=0.877$). Also, the difference in the response to infection by *E. lata* when the pathogen was introduced on Day 2 and Day 7 was not significant ($P=0.216$).

Results for isolation of *E. lata* from 2-10 cm below the point of inoculation from canes treated with the pathogen 2 days after the antagonist in the second trial are shown in Figure 5.1. *E. lata* was isolated from 100% of the canes treated with SDW, but from less than 30% of the canes treated with the antagonist. The differences in the probabilities of re-isolation of *E. lata* (for 2 cm below the point of inoculation) from canes treated with viable antagonist and the control treatments (Table 5.16) were highly significant ($P<0.001$), with the probabilities being 0.81, 0.81 and 0.94 respectively.

Re-isolation of *T. harzianum* was carried out only for the cuttings inoculated with the pathogen on day 2. Many of the wood samples (87.5%) treated with Trichoseal® containing the 7 strains of *T. harzianum* yielded *T. harzianum* at a distances of up to and including 10 cm below point of inoculation, 12 weeks after the pruning wounds were treated (Figure 5.2). *T. harzianum* strain 1 was re-isolated from canes treated with viable *T. harzianum* at 2 to 10 cm below the point of inoculation but the frequency of recovery generally decreased with distance from the point of inoculation. *T. harzianum* was not recovered from any of the controls treated with gamma-irradiated *T. harzianum* strain 1 in Trichoseal® base, gamma-irradiated Trichoseal®, or SDW.