1 Introduction

1.1. The Australian cotton industry

Cotton production in Australia had its beginnings shortly after the arrival of European settlers in 1788. Production rose and fell over the next century and a half, with 17 000 bales produced in 1934 (Cotton Australia 2008). The following 20 years saw a major decline in production, and the industry as we know it now really only commenced in 1961 with a commercial, irrigated crop planted at Wee Waa in northern NSW after completion of Keepit dam in 1958 (Cotton Australia 2008). The areas under production expanded into new valleys with the completion of several dams providing irrigation water. By 1985, production had reached over one million bales. In 2005, production peaked at 2.9 million bales with production in the following year decreased due to drought conditions (Cotton Australia 2008).

The industry in Australia is a world leader. Yields per hectare are the highest in the world. In 2006/07, Australia produced 1792kg ha^{-1}, while the world average was only 747 kg ha^{-1} (International Cotton Advisory Committee 2007).

1.2. Challenges for cotton growing

Crop growers face a range of challenges throughout the growing season. In order to overcome some of these challenges, the chemical production industry has developed a large range of products that help farmers defend their crops. For example, WEEDpak (published by the Cotton CRC in August 2002) contains a list of more than 70 products from over 20 different chemical companies that are used to treat a range of problems in cotton. As we move into the 21st century, there is more awareness that chemical treatments are not always the best answer for sustainable agriculture in a healthy environment. Both farmers and industry groups are recognising that an integrated approach that incorporates a range of management strategies that reduce reliance on the application of chemicals will be more sustainable in the long term, providing both environmental and economic benefits (Cotton Research and Development Corporation Annual Report 2004 2005).

The aim of this project was to develop a treatment approach for a significant soil-borne fungal pathogen, *Thielaviopsis basicola*, with an emphasis on microbial control and non-synthetic chemicals applied to cotton farming in north-western and central western New South Wales, Australia.

1.2.1. The fungal disease challenge

There are a number of fungi that can negatively impact cotton production including species from genera including *Rhizoctonia*, *Pythium*, *Verticillium*, *Fusarium* and *Thielaviopsis* (Allen et al. 2008). A pathogenic strain can have a limited host range, for example, *Fusarium oxysporum* f.sp. *vasinfectum* (FOV) causes disease in cotton but not wheat (Allen et al. 2008), even though both crops may be grown in the same fields.

Soil-borne diseases can be particularly difficult to control, as the species that cause them often produce long lasting spores that can survive in the soil for more than one season (Inglis & Cook 1986; Sewell & Wilson 1966; Tsao & Bricker 1966) and effective fungicides are not always available. In these cases solutions that do not rely on synthetic chemicals must be sought.

In some cases, non-chemical management of a disease proves to be highly effective. Management of *F. oxysporum* f.sp. *vasinfectum* (FOV) provides an example of a successful non-chemical strategy. When FOV was recognised as a serious threat to cotton production in Australia, industry stakeholders formed a working group to co-ordinate research and working closely with breeders, developed varieties that were more resistant to the disease (S. Allen, pers. comm.). Varieties were scored according to their ability to resist infection (Allen et al. 2008) and this F-rank was made available to growers (Disease Ranks 2008) who could incorporate this into their decisions when choosing varieties. When the disease was known to be present on farm, growers could select varieties with high F-ranks. This proved to be a very successful strategy in reducing the incidence of FOV in crops (Nehl et al. 2007), however, the success in managing FOV cannot be repeated for diseases where there is no varietal resistance.

Where varietal resistance is not available, genes that confer resistance to a particular disease or threat can be introduced into plants using bio-technology. In 2006/07, over 95% of cotton growers in Australia planted varieties that have been genetically modified (Cotton-Australia 2009) with genes isolated from a soil bacterium, *Bacillus thuringiensis*, encoding proteins toxic to the larvae of *Helicoverpa* sp. This genetic technology has been incorporated into many of the commercially available cotton varieties and has led to large reductions in the use of endosulfan, a pesticide that was used to manage the *Helicoverpa* (Cotton Research and Development Corporation Annual Report 2004 2005) and increased yield compared to non-Bt cotton varieties (Pray et al. 2002; Pyke 2007). While this technology has proven to be extremely successful for cotton production, biotechnology solutions such as these are not without their problems. The first is that while some consumers are willing to accept genetically modified organisms (GMOs), there is still a strong prejudice from certain sections of society against GMO foods (Francis 2006). Genetically modified cotton is currently approved for use in Australia (OGTR 2006), however, each new GM trait still needs to be approved by the Office of the Gene Technology Regulator before release (OGTR 2008), leaving open the possibility that regulation will prohibit its use. Another potential disadvantage of GM technology is limited transferability. A protein encoded by a gene inserted into the cotton genome may be useful for protecting against the same disease in another crop, but transferring a gene into a new crop or even a new variety will take time. For example, it was suggested in 2001 that a modified gene based on a radish defence protein (Rs-AFP2) could be inserted into plant genomes (including cotton) to inhibit disease (Posthuma et al. 2000) but as yet, no such modified varieties appear to be available on the market. Where genetic resistance is currently unavailable, other mechanisms for disease control need to be sought.

1.3. Black root rot in Australian cotton

Black root rot in cotton caused by *T. basicola* was first described in Australia in 1989 (Allen 1990). It is a seedling disease that attacks the roots of newly germinated seeds and leads to characteristic blackening of the tap root (Figure 1).

The disease does not usually kill seedlings, but leads to decreased yields through early season stunting (Hake et al. 1985; King & Presley 1942; Mathre et al. 1966).

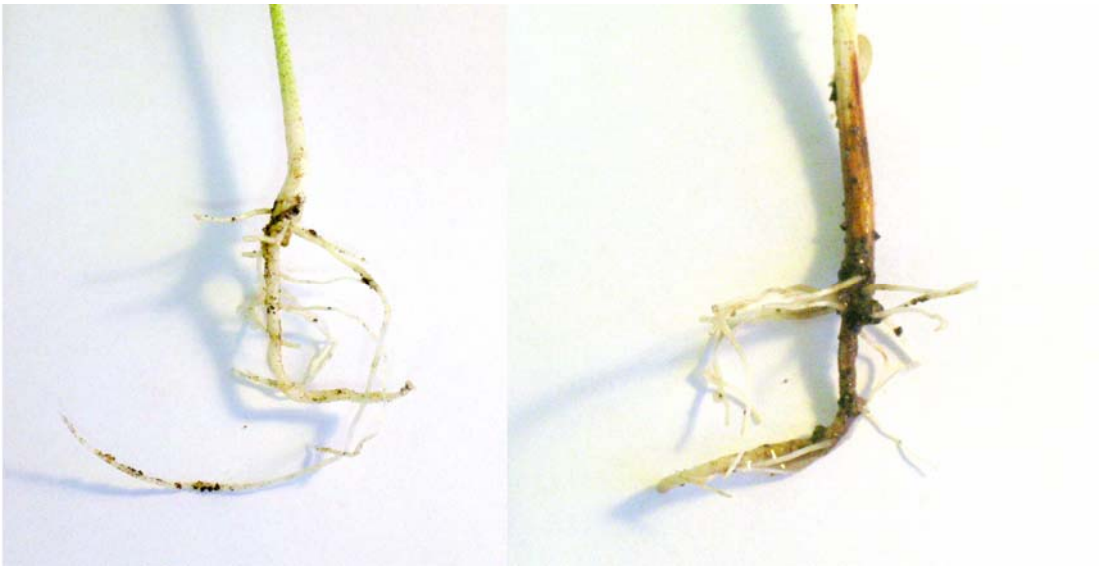


Figure 1: Healthy (left) and diseased (right) cotton roots. The characteristic blackening of the roots (especially the tap root) due to *T. basicola* infection is clearly visible in the right image.

There does not appear to be any evidence linking the initial outbreak in cotton to a specific source, however the pathogen had previously been reported by Simmonds (1966), Sampson and Walker (1982) and Warcup and Talbot (1981) in Australia on a variety of plants, including tobacco, bean and radiata pine (cited in (Allen 1990). Since 1989, the disease has spread rapidly in cotton growing areas and in 2003 was found on 29 out of 30 farms surveyed in the Macintyre, Gwydir, Namoi and Macquarie valleys (Nehl et al. 2004). There are currently no effective treatments for infestation.

1.3.1. Current disease management options

Tobacco growers have been able to select plant varieties that have at least partial resistance to *T. basicola* infection due to the presence of transferable resistance genes in some *Nicotinum* species (Gayed 1969; Wilkinson et al. 1991), with research into mechanisms of resistance being carried out at least as early as 1927 (Conant 1927). For industries such as cotton production, varietal resistance is

currently unavailable (S. Allen, pers. comm.) and the area under production makes some other potential solutions physically or economically impractical (discussed below). Until varietal resistance to this disease becomes available, other mechanisms need to be used to minimise disease impact.

There are several ways growers can manage an infestation of *T. basicola*, as listed in the Integrated Disease Management manual available from the Cotton CRC (2008). The primary recommendations are planting when soil temperatures are above 16°C and rising and pre-irrigating soil, rather than watering soon after planting (Allen et al. 2008). These management options do not treat infestation, but gives plants the best chance to outgrow the disease symptoms quickly. Other potential management options suggested in the manual include summer flooding, rotating with non-host crops and planning for later picking to allow for delayed maturity. Recently, acibenzolar-S-methyl (commercially known as Bion™) has been suggested as a seed treatment to induce host resistance against black root rot, though results in Australia have been mixed (Mondal et al. 2005).

Most of these management options are not treatments, except flooding which is usually not practical due to limited water availability (Current Drought Situation 2008) and application of Bion™ which only provides limited protection, by way of minimising disease impact (Mondal et al. 2005). The practicality of the remaining options can be limited. Allowing for planting after soil temperature is at 16°C and rising, or picking later, is weather dependent and rotation with non-host crops limits growers choices.

Implementation of the “Come clean, go clean” recommendations, involving washing down vehicles entering and leaving the farm with an anti-fungal detergent can limit transfer of the disease to new areas, however, even though many growers are using these practices, the disease has still spread rapidly in the last decade and is now found on the majority of regularly surveyed farms in the major cotton growing regions in Australia (Nehl et al. 2004). As this disease is now so widespread and there are only limited options for its control, an active disease management and treatment regime is required.

1.4. Thielaviopsis basicola

In order to propose a new strategy for dealing with *T. basicola* infestation it is important to have an understanding of the organisms biology and life cycle.

T. basicola is a widespread, filamentous soil-borne fungus. Strains of *T. basicola* infect a wide variety of plants across a range of plant families, including many commercially valuable plants such as tobacco, cotton and lettuce as well as ornamental plants such as pansy and pine. *T. basicola* has been characterised as a fungal pathogen since at least 1876 (Johnson 1916). It has been detected in soil in many countries including Europe, America (Yarwood 1981) and Australia (Nehl et al. 2004) and several researchers have produced lists of host range over the last century (Johnson 1916; Yarwood 1981).

Since the initial description of this organism was published in the late 1800s (Johnson 1916), it has been known under several names. *Thielavia basicola*, *Torula basicola*, *Thielavia renominata* (Paclt 1960), *Chalara elegans* (Nag Raj & Kendrick 1975) and *Thielaviopsis basicola* have all been suggested, with *Thielaviopsis basicola* now the most common name in use, although some researchers still use *Chalara elegans* as a synonym (O'brien & Davis 1994; Punja & Sun 1999).

T. basicola isolates from different geographical regions and different host plants exhibit a high degree of genetic diversity (Punja & Sun 1999). Spontaneous morphological changes also occur from time to time in laboratory cultures (Huang & Patrick 1971), one obvious change causing reduced pigment production and sporulation (Punja 1993). These factors have led to many different *Thielaviopsis* species being suggested, however, Paulin-Mahady, Harrington and McNew (2002) have conducted a molecular analysis of many of the proposed species and concluded (in part) that *T. basicola* is in a phylogenetically separate group with three other proposed species, all of which are soil-borne, and that the production of spores in chains is considered to be a distinguishing feature of *T. basicola*, separating it from the other three. This is in agreement with Punja and Sun (1999),

who determined that although there is a high degree of genetic variability within isolates of this species, this does not justify the creation of multiple species within the morphological variants.

There has been a substantial quantity of the research into *T. basicola* spanning the last century focused on its importance to commercial crops, especially tobacco (*Nicotinum tobacum*) (Conant 1927; Gayed 1969) and cotton (*Gossypium hirsutum*) (King & Presley 1942; Mathre et al. 1966). In both of these crops infection can cause yield losses (Nehl et al. 2000; Stover 1950) reducing farm profitability. While some research has focused on yield effects, other research has focused on the physiological and histological characteristics of *T. basicola*, such as responses to pH and temperature (Lucas 1955), persistence in soil (Papavizas & Adams 1969; Tsao & Bricker 1966), how spores germinate (Mathre & Ravenscroft 1966) and early infection processes (Hood & Shew 1997a).

T. basicola can be easily grown in culture (King & Presley 1942; Yarwood 1946). In the soil where it is naturally found, growth is dependent on the presence of a host plant and so this species is classified as a hemibiotroph (Hood & Shew 1997a; Mims et al. 2000). It is interesting to note that a germinating hyphae, (or germ tube) will pass through dead root tissue without any apparent differentiation, suggesting that the process of infecting root tissue initially requires a living cell (Hood & Shew 1997a).

Using a variety of microscopy techniques, detailed descriptions of the infection process in root tissue have been published for pansy (Mims et al. 2000), tobacco (Hood & Shew 1997a) and cotton (Mauk & Hine 1988). Infection of a host begins with the germination of a spore present in the soil. The spore can be either of the two types, the relatively short lived endoconidia or thick walled chlamydospores that are generally considered to be the propagule that survives between seasons. A germ tube extends through the wall of the spore and elongates. When it comes in contact with a host root it may either continue to grow over the surface without penetrating the cell (non-inductive contact), or it may cease elongation and thicken slightly before extending a thinner penetration hyphae through the host cell wall (inductive contact) (Mims et al. 2000).

The plant cellular response to the contact with the germ tube consists of cytoplasmic streaming towards the contact site and in some cases, formation of a papilla (a thickening of the cell wall at the point of contact). Papilla formation seems to be dependent on plant species. A papilla has been observed in pansy roots (Mims et al. 2000) but not in non-resistant tobacco (Hood & Shew 1997a). When a papilla does form, in non-resistant plants, it does not prevent the entry of the penetration hyphae, which continues to grow into and then through the papilla. Once inside the cell, the tip of the penetration hyphae expands and then differentiates, forming an intracellular hyphal cell. During this early infection phase, the host cell produces callose material around the point of penetration, but this fails to prevent the invasion and necrosis of the host cell occurs within hours (Hood & Shew 1997a).

After penetrating the cell wall and formation of the intracellular hyphal cell, two differentiated types of hyphae are then formed within the host tissue; constricted hyphae that are identified by a narrowing at the septum, which grow to fill the cell (Christou 1962) and unconstricted hyphae that penetrate neighbouring cells (Mauk & Hine 1988). As the infection progresses, long and unconstricted reproductive hyphae form and produce both endoconidia and chlamydospores.

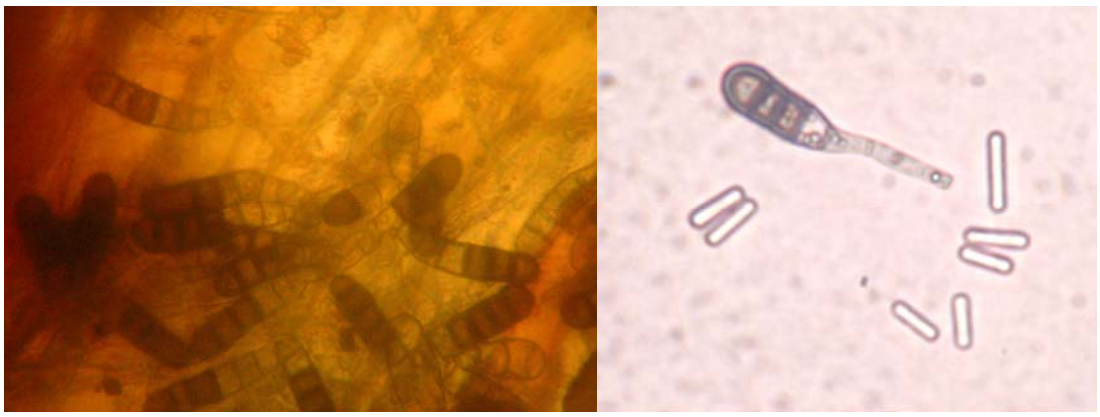


Figure 2: Left image; *T. basicola* chlamydospores produced on infected cotton roots. Right image; A suspension of endoconidia (clear rods) and chlamydospores obtained from *T. basicola*.

Invasion of host root tissue by *T. basicola* is usually limited to the outer layers of tissue, with the stele and endodermis usually remaining uninfected. As the host matures, increased growth often leads to sloughing off of the dead tissue and the development of new uninfected tissue, although in some cases infection can persist into mature tissue (Mauk & Hine 1988). As dead tissue is discarded, it also releases large numbers of spores into the surrounding soil making it is possible for plants to become reinfected throughout the season and adding to the level of inoculum in the soil (King & Presley 1942) (Figure 3).

The process of chlamydospore formation has been well described (Riggs & Mims 2000), though the formation of endoconidia has not. In the process of forming chlamydospores, a sporogenous cell buds from a hypha. A nucleus enters the cell and a septum forms dividing the two cells. Mitosis occurs and another septum forms, producing two cells. This process is repeated, resulting in a chain of 2-7 cells. Once the chain has finished forming, the cells develop the thick melanised cell wall that differentiates them as chlamydospores (Riggs & Mims 2000). In addition to the cell wall of each individual spore, a single cell wall also surrounds the spore chain as it is forming. It has been speculated that this outer cell wall protects the chlamydospores while they are developing (Delevecchio et al. 1969).

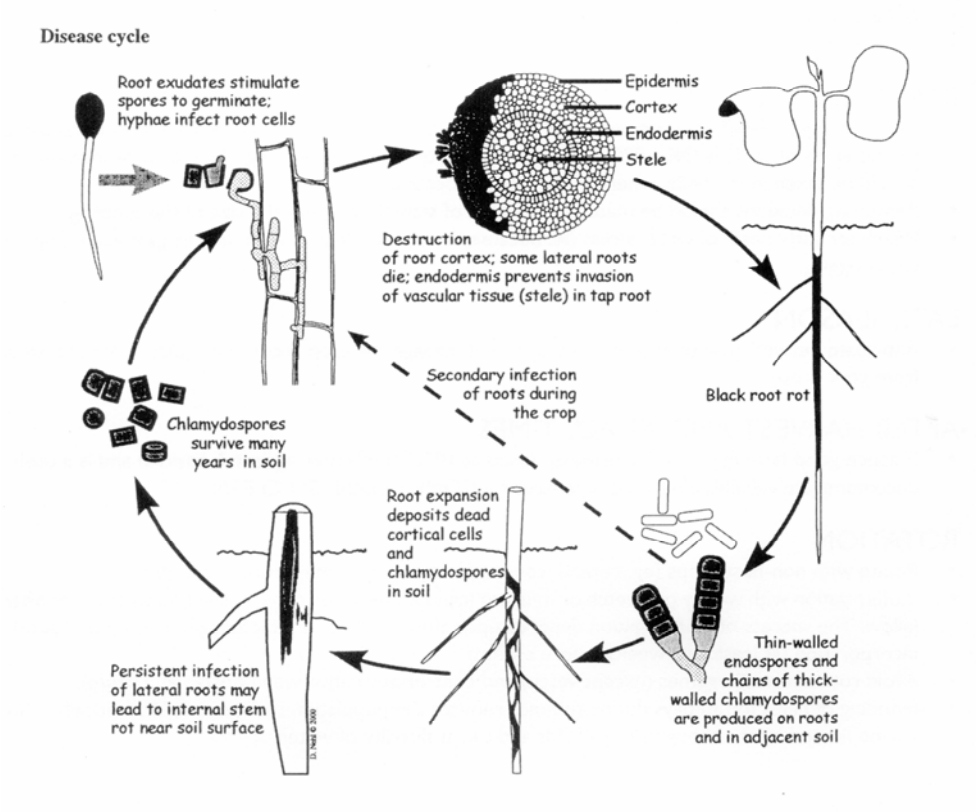


Figure 3: Disease cycle of *T. basicola* on cotton roots. Image from (Allen et al. 2008)

New management options could target different stages of the *T. basicola* lifecycle. Areas that may be promising to target could be the recognition and infection of host plants, growth rate of the fungus or the survival of the propagules in the soil. It may be possible to prevent *T. basicola* recognising and infecting host cotton plants using soil bacteria, slow the growth rate of the fungus with an anti-fungal agent or competing micro-organisms, or induce dormant spores to germinate in the absence of host plants, leading to reduced pathogen load in the soil.

1.5. Non-synthetic disease control options for field crops

There is an increasing demand in the community for products that are organic, with sales of organic products increasing rapidly (BFA 2008; *Organic Exchange 2007 Annual Report* 2008). There is also increased uptake of integrated approaches to pest management by cotton growers looking to improve

environmental sustainability (Cotton Research and Development Corporation Annual Report 2004 2005). This means that the development of systems that treat diseases without the use of synthetic chemicals is becoming more important. After examining the life cycle of *T. basicola* it is now appropriate to look at the range of non-chemical options already available for treating plant diseases.

1.5.1. Rotation

In seasonal climates, such as in NSW, cotton can only be grown in the warmer months, so a rotation system will normally be used. Rotations can include the use of income producing crops such as wheat or legumes, a bio-fertiliser crop that will be ploughed in to increase soil nutrient levels for the following crop or fallows where no crop is planted (D. Nehl, pers. comm). When considering candidate rotation crops, the presence of a disease such as black root rot will limit the available options, as selecting a crop that can host the disease, such as soy bean, will lead to increased inoculum levels and higher disease incidence in subsequent cotton crops (Mondal et al. 2004). By selecting a non-host or fallow in rotation, the level of inoculum in the soil should not increase over the winter season and depending on the ability of the inoculum to survive in the absence of a host, may even decrease. For black root rot, due to the long surviving chlamydospores (Tsao & Bricker 1966), the disease will reappear next time a host crop is planted. In a long term rotation trial, Nehl *et al.* (2004) concluded that rotation with a non-host such as wheat delays progression of the disease but does not prevent it. In that study, the number of previous cotton crops was determined to be the single most significant factor in predicting the severity of disease in an individual season, regardless of the rotation crop used. Careful selection of rotation crops is therefore important to avoid increasing inoculum levels, but has limited use in intensively farmed fields as a preventative measure.

1.5.2. Biofumigation

This management technique involves growing a crop that is ploughed into the soil. The breakdown products of the crop are toxic to the fungal spores and can lead to decreased disease symptoms in the following cotton crop. Several species have been tested in trials including Indian mustard (Nehl et al. 2000) and vetch

(Candole & Rothrock 1998; Rothrock et al. 1995) with mixed results. Candole & Rothrock (1998) suggested the mechanism of suppression due to incorporation of vetch was due to the production of ammonia during breakdown in soil, causing a reduction in viability of chlamydospores, which are the primary inoculum found in soil.

There are some significant disadvantages to the use of biofumigation as a disease treatment measure. Firstly, growers may be resistant to planting crops that do not produce an income. To plant and maintain a bio-fumigant crop will cost money that needs to be recouped through higher yields in the subsequent cotton crop. There needs to be a significant benefit demonstrated before this method would be widely accepted. One potential solution to this is to choose bio-fumigant crops that give a nitrogen benefit, reducing the need for fertilisers in the following crop. Unfortunately, some legumes used for this purpose are highly susceptible to black root rot (Mondal et al. 2004). The limited availability of water can also make the planting of additional non-cash crops more prohibitive (Climate Averages 2009).

Secondly, although the bio-fumigation crop may reduce the incidence of *T. basicola* infection, it may be a host for other diseases. This has been shown to be an issue for the use of Vetch in Australia leading to increasing incidence of *Pythium ultimum* and *R. solani* in the United States (Candole & Rothrock 1998).

1.5.3. Induced resistance

Although plants do not have an immune response like that of animals, they do have natural defence mechanisms, both passive and active, that prevent infection by a pathogen. Some mechanisms include producing antimicrobial compounds such as the defensin proteins released from some seeds upon germination (Broekaert et al. 2000), strengthening cell walls at the site of an attack, forming a papilla, or modifying cells surrounding a wound with lignin to isolate an infected cell (Moerschbacher & Mendgen 2000).

In 2004, Edreva presented a review of induced resistance research and defined induced resistance as “an increased expression of natural defence mechanisms of

plants against different pathogens provoked by external factors" (Edreva 2004). By providing appropriate "external factors" (commonly referred to as elicitors) it may be possible to pre-activate a plant to increase the speed or effectiveness of its defence responses. Some of the elicitors reported include the use of isonicotinic acid to reduce verticillium wilt in cotton (Colson-Hanks & Deverall 2000) and benzothiadiazole (BTH) treatment of grapes to reduce nematode infestation (Owen et al. 2002). In one trial specific to cotton, seeds were soaked in a solution of benzothiadiazole (BTH) prior to planting, resulting in a small decrease in observed disease symptoms (Mondal et al. 2000). Acibenzolar-S-methyl (commercially known as Bion™) has also been suggested as a seed treatment to induce host resistance against black root rot. Results in Australia have been mixed, with disease suppression of up to 33% observed (Mondal et al. 2005).

In some cases, bacteria can be an elicitor. *Arabadopsis thaliana* plants have been shown to be protected from *Pseudomonas syringae* (a leaf pathogen) by application of *Chryseobacterium balustinum* to the soil in which they are grown (Ramos Solano et al. 2008). Treatment of cucumber seeds with *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166 can also induce resistance to a bacterial leaf disease (Liu et al. 1995).

The induced resistance response can be local or systemic (Van Loon 2000). It is the systemic acquired resistance (SAR) response that is the most promising for application to crops. Systemic responses, where an elicitor applied to one part of the plant causes a reduced susceptibility to attack throughout the plant, could allow a foliar application of an elicitor to protect the whole plant, including the root system that may be attacked by a soil borne pathogen. As an example, cell wall components of certain *Pseudomonas fluorescens* strains have been shown to induce resistance to infection of radish roots by *R. solani* when applied to the cotyledon (Leeman et al. 1995). While potentially effective, this type of approach may have limited use against a disease such as black root rot that attacks seedlings even before they have emerged.

1.5.4. Soil amendments and bio-fertilisers

Addition of amendments to soil as a method of increasing production is an old idea. During the 20th Century, a number of books have been published guiding growers on the use of composts as a substitute for synthetic fertilizer. Harwood (1990) mentions several in his history of sustainable agriculture.

Organic amendments can be used for more than just enhancing production. They can also be used to reduce or prevent incidence of disease. In a review of the mechanisms of disease suppression by composts, Hoitink and Fahy (1986) present some examples of the use of composted bark to suppress a brown rot of yams in Japan and a *Pyhtophtora* disease of strawberries. Liquid swine manure has also been shown to suppress *Verticillium dahliae* in potato crops (Lazarovits et al. 2001). These type of soil amendments are often applied at high rates (tonnes per Ha) so when they can be sourced cheaply, such as when using a waste product from another industry, they can be an effective option.

In addition to waste products and composts, some bacteria can be considered as bio-fertilisers. In one large scale field trial over multiple seasons, application of a multi-strain inoculant saw rice yield increases of greater than 10% compared to application of urea (Nguyen et al. 2003). Another trial applied a seed treatment containing a rhizobia, mycorrhiza and *Pseudomonas* species to faba bean seeds and found that combination treatments significantly increased numbers of seed pods in mature plants compared to application of the recommended dose of NPK fertiliser (El-Wakiel & El-Sebai 2008).

1.5.5. Suppressive soils

Some soils appear to have a natural ability to suppress the development of *T. basicola* infections in tobacco (Stutz et al. 1989; Stutz et al. 1985) and anecdotally in cotton (S. Dreissen, pers. comm.). Even though there may be a relatively high level of inoculum in these soils, the level of disease symptoms are lower than expected. There are two categories of factors that may contribute to the suppression of soilborne fungal diseases, physical (abiotic) factors and biological (biotic) factors.

Abiotic suppression

Abiotic suppression is due to the physical characteristics of soil such as temperature, pH, water content or availability of certain chemical components such as aluminium and nitrogen. Dry conditions, especially in combination with higher temperatures, have been shown to reduce the level of infection by *T. basicola* in cotton plants (Rothrock 1992). Fungi, including *T. basicola*, can grow in a wide pH range in culture (Lucas 1955). Low pH has been shown to have a negative effect on the growth of *T. basicola* in soil (Meyer & Shew 1991a, 1991b), however, some research suggests that this may not be due to pH directly rather due to an increase in solubility, and therefore toxicity, of aluminium (Meyer et al. 1994). In either case, the suppression of *T. basicola* in conditions of low pH would be considered an abiotic factor.

Nitrogen fertility has been implicated in suppression of black root rot in tobacco via a host mediated response that limits production of spores after infection. Plants exposed to high levels of NH_4 produce putrescine, which is toxic to *T. basicola* (Harrison & Shew 2001). This is a more complex example where the mode of action of an abiotic factor (NH_4 availability) has been determined and leads to a biological response.

Abiotic factors may be incorporated into management plans. It is recommended that cotton in Australia is planted into watered soil rather than watering after planting, based partly on the results of Rothrock (1992) showing reduced disease at lower water potential.

Biotic suppression

Some soils contain micro-organisms that can suppress a particular disease. This suppression can be through direct antagonism by production of a toxic metabolite (antibiosis) (Paulitz et al. 2000; Raaijmakers et al. 2002) inducing resistance in the host (Colson-Hanks & Deverall 2000; Mondal et al. 2005; Mondal et al. 2000), or by competition for limited nutrients (Whipps 2001).

Micro-organisms have been isolated from soil where a disease causing organism is known to be present but disease levels are lower than expected. Barnett et al. (2006) observed unexpectedly low levels of infection by *R. solani* AG-8 in a wheat growing field in South Australia. It was shown that this suppression disappeared when soil was sterilised, but when certain isolated strains were allowed to recolonise the soil, the suppressive effect returned. Another study isolated multiple Psedomonads from soil that was naturally suppressive to black root rot in tobacco and found that addition of some of the strains to soils conducive to black root rot reduced disease levels (Stutz et al. 1985). In other cases, researchers have taken strains that suppress disease causing micro-organisms in-vitro and then applied them to field situations. Siddiqui & Shaukat (2002) took this approach in selecting two Pseudomonads that inhibited multiple fungal pathogens in plate tests and applying them to field grown tomato plants to improve shoot growth in plants under disease pressure from *Macrophomina phaseolina*, *Fusarium solani*, *Rhizoctonia solani* and *Megloidogyne javanica* (a root-knot nematode).

It can be a significant amount of work identifying which of the micro-organisms in the soil are causing a suppressive effect and once isolated, selecting species/strains that will be effective when applied to field conditions. Knudsen et al. (1997), reviewed several methods of selection and concluded that the most reliable method in relation to identifying field relevant bio-control agent is field testing. This is a logical conclusion, as organisms have been isolated that have shown a suppressive effect during *in-vitro* testing then fail to perform under more natural conditions (Reddy et al. 1994) or are effective only in some soil types (Stutz et al. 1989). The difficulty with field testing is that there are limited opportunities each year to obtain data. Thus, it is important that care is taken with the methods and systems used to screen potential control mechanisms to strike a balance between reliable and reproducible results, and expediency. In addition, it is important to note that the ability of soil to suppress a particular disease will often not extend to other diseases and so the suppressive ability of a soil will likely need to be considered on a per disease basis (Oyarzun et al. 1998).

1.6. Management strategies to be examined in this study

By combining the knowledge of the life cycle of *T. basicola* with what research has already been done, multiple possible strategies for controlling black root rot have been selected for investigation in this study. Any proposed system not only has to be effective in-vitro, but has to be able to be applied on a large scale and be suitable for Australian conditions.

1.6.1. Inoculation of soil with non-indigenous bacteria

Many species of bacteria have been used to promote plant growth and inhibit disease. Species of *Pseudomonas*, *Bacillus* and *Serratia* have all been shown to inhibit plant diseases in a variety of ways, such as induction of resistance to fusarium wilt in radish caused by *Pseudomonas fluorescens*, (Leeman et al. 1995), in cucumber (*C. obiculare*) to bacterial angular leaf spot caused by *Pseudomonas putida and Serratia marcescens* (Liu et al. 1995) and reduced damping off caused by *R. solani* when plants were exposed to certain *Bacillus* strains (Jetiyanon & Kloepper 2002). It is useful to note that under Australian standards, any naturally occurring organism may be used in organic farming provided it has not been genetically modified (*National Standard for Organic and Bio-dymanic Produce* 2005). This means that use of such systems for disease suppression would be acceptable for organic cotton farming and therefore considered to be sustainable.

A group of three bacteria, *Pantoes agglomerans*, *Exiguobacterium acetylicum* and *Microbacterium* sp., were isolated from wheat growing soil in Avon, South Australia and have been shown to suppress disease progression caused by *Rhizoctonia solani* AG8 (Barnett et al. 2006). Although a mechanism for the observed suppression of *R. solani* was suggested, it was not further tested. In addition to suppression of *R. solani* in wheat, strains of *P. agglomerans*, *E. acetylicum* and *Microbacterium* have individually been implicated in inhibition of multiple plant pathogens in a range of crops including Anthurium (lily), cucumber, pea, apple and orange (Fukui et al. 1999; Postma et al. 2005; Selvakumar et al. 2009; Stockwell et al. 2002; Teixidó et al. 2001).

It is hypothesised that strains of these bacteria may have the ability to suppress *T. basicola* leading to two of the aims of this study. The first is to test the activity of these bacteria against *T. basicola* under laboratory and field conditions. If a suppressive effect is observed, the mechanism for the suppression will be investigated, testing for direct inhibition effects, either singly or in combination. As there are examples of strains from some of these species causing disease, the laboratory trials will ensure the strains used do not increase disease pressure before field application begins.

Another soil bacterium that has been shown to have beneficial effects in other crops is *Azospirillum brasilence*. This motile soil bacterium can form a close association with plant roots, including cotton, and some studies have linked this species with growth promoting effects through the fixation of nitrogen and production of a range of growth promoting chemicals including indole acetic acid and gibberellin (Tien et al. 1979). *Azospirillum* species have been applied to many crops in a range of climates around the world and a review in 1994 concluded that of the published data from the preceding 20 years, 60-70% of applications resulted in increased yields in field trials in crops including maize, wheat, sorghum and millet (Fulchieri & Frioni 1994; Okon & Labandera-Gonzalez 1994). Application of *A. brasilense* to tomato leaves has recently been shown to be effective in reducing leaf spot in tomato plants by out-competing the pathogen (Bashan & De-Bashan 2002) and in *Arabadopsis thaliana* when applied to soil, possibly through stimulation of a systemic host response (Ramos Solano et al. 2008).

Since *Azospirillum* strains have been shown to have a growth promoting effect, and *T. basicola* causes stunting of growth, it may be possible that application of *Azospirillum* will reduce the symptoms of black root rot by increasing plant growth or competing with *T. basicola* in the rhizosphere. Previous experiments in this laboratory have investigated whether a particular strain, *A. brasilence* SP7, can reduce disease symptoms and in cotton plants have shown a reduction in root blackening when plants were pre-germinated and inoculated with this strain prior to planting in infested soil (Pereg & Mijhalovic, unpub). The experiments conducted in this study will investigate whether this disease suppressing effect will

still be present when this strain is applied to seed, rather than seedlings, and if the effect can be observed under field conditions.

1.6.2. Isolated anti-fungal proteins as a planting treatment

Plants have a range of methods by which they can protect themselves from attack by pathogens. There are physical barriers to entry such as papilla formation and lignification of cells (Moerschbacher & Mendgen 2000). There are also chemical methods such as release of simple compounds including superoxide and hydrogen peroxide (Jabs & Slusarenko 2000; Lamb & Dixon 1997), or production of more complex anti-microbial compounds like proteins (Broekaert et al. 2000).

Many anti-microbial proteins have been described sourced from plants, bacteria, fungi and animals (Selitrennikoff 2001). An1 isolated from *Atriplex nummularia* (saltbush) inhibits *Rhizoctonia solani*, *Verticillium dahliae* and *T. basicola* in-vitro (Last & Llewellyn 1997). An African insect, *Rhodinus prolixus*, produces defensins that prevent it becoming infected by Chagas disease, for which it is a vector (Lopez et al. 2003). Magainin 2 produced by the African clawed frog inhibits *T. basicola*, *R. solani*, *V. dahliae* and *Pythium ultimum* in-vitro (Kristyanne et al. 1997).

Some anti-microbial proteins have been suggested as having potential for treating plant diseases by inserting the genes into the target plant. For example, a 5.5kDa peptide isolated from alfalfa, alfAFP has been successfully expressed in potato to inhibit *V. dahliae, Alternaria solani* and *Fusarium culmonorum* (Gao et al. 2000). There are advantages and disadvantages to this approach. Once a gene is successfully incorporated into the genome and is being expressed, the gene product can potentially protect the entire plant for its whole life. Use of the Cry genes, originally isolated from *Bacillus thuringiensis*, in cotton to protect the plant from bollworm (*Helicoverpa* sp), is a successful example of this type of approach (Pray et al. 2002; Pyke 2007). Expression of the inserted genes in plant tissue leads to production a protein that is toxic to the *Helicoverpa* larvae, thus protecting the plant from attack and limiting the ability of this common cotton pest to reproduce (Perlak et al. 2007).

For a disease such as black root rot, plants need protection only in the first few weeks post planting as disease levels decline later in the growing season when temperatures increase (Rothrock 1992). This means ongoing production of the anti-microbial protein in plant tissue is unnecessary. In addition, GM technology has potential problems of acceptance and time to implement (see section 1.2.1). Production of the protein outside the plant and addition to the soil at planting may provide sufficient protection. This also removes any potential metabolic load borne by the plant to produce the protective protein itself and allows the protective effect to be applied to other crops and varieties without waiting for breeding to introduce the appropriate genes.

This study examines a specific protein originating from *Raphanus sativus* (radish). The protein, designated Rs-AFP2, has been shown to suppress multiple fungal pathogens at low concentrations in-vitro, including two strains of *Fusarium oxysporum* and a strain of *Verticillium dahliae* (Vilas Alves et al. 1994). A crude extract from *R. sativus* seeds has also been shown to suppress *T. basicola*. Combining these two sets of data, it is hypothesised that protein Rs-AFP2 may suppress *T. basicola*. This specific protein was selected for a number of reasons in addition to its ability to suppress pathogenic fungi. Rs-AFP2 has been previously expressed in a yeast expression system (Terras et al. 1995), making it easier to design experiments utilising relatively pure protein, without the use of complex purifications steps. Commercially available yeast expressions systems are available that can produce in excess of 50mg of protein per litre (NEBS). The protein is also quite stable, surviving heating to 100°C (Terras et al. 1992). Heat stability is an important factor as daytime temperatures in cotton growing regions can exceed 40°C (*Climate Averages* 2009) and any product would potentially need to be stored on-farm prior to application.

The first set of experiments will confirm the activity of *R. sativus* extract against *T. basicola*. The AFP2 gene will then be reconstructed and a yeast expression system will then be constructed to produce pure protein. The purified protein will be tested in anti-fungal assays first in culture and then in plant systems.

1.6.3. Soil amendments as a decoy

Manure, crop residues and other organic wastes can all be used in agricultural systems to improve soil, provide nutrients (Nustorova et al. 2006) and suppress soil-borne disease (Hoitink & Fahy 1986; Lazarovits et al. 2001). These kinds of amendments are often abundant and their use as a fertiliser can assist in solving the problem of disposal of waste from other industries.

Some common substances have also been suggested as inducers of germination for *T. basicola*, including certain root exudates, such as fatty acids (Papavizas & Kovacs Jr 1972), lecithin, soybean protein, zein, yeast extract and chestnut tannin (Papavizas & Adams 1969). As *T. basicola* requires living tissue to reproduce in soil (Hood & Shew 1997a), inducing germination of spores in soil some time prior to planting may significantly reduce the inoculum load in the field.

As natural soil is a complex system of chemical and biological factors, it can be difficult to determine whether the addition of a chemical in a natural soil system stimulates germination directly or acts by stimulating another micro-organism that uses it as a substrate. In laboratory studies, it is possible to eliminate the effect of other micro-organisms through the use of sterilised soil, however, these results may not successfully translate when applied to field conditions (Lindow 1988b; Reddy et al. 1994) so care must be taken with any positive result not to extrapolate without first testing under natural conditions. Regardless of the mechanism, it may be possible to utilise some common substances for disease suppression.

In order to be useful in the field, such amendments have to be readily available in large quantities as they may need to be applied at high rates per hectare (Hoitink & Fahy 1986; Lazarovits et al. 2001). Some products have been tested against *T. basicola* in the past including fatty acids (Papavizas & Kovacs Jr 1972) or chestnut tannin (Papavizas & Adams 1969) but may not be practical as they are not available in large quantities. Other suggestions include yeast extract, zein and soy protein (Papavizas & Adams 1969). These last three are available in large quantities, as they are all used commercially in food production (soy flour is

primarily composed of soy protein, zein is used in the confectionary industry as a glazing agent and yeast extract is used industrially in the production of products such as Vegemite™).

Pot trials using applications of soy protein, zein and yeast extract to fallow soil infested with *T. basicola* will be used to examine the effect of application of these substances on disease levels in subsequently planted cotton.

Allen, S.J. 1990, '*Thielaviopsis basicola*, a new record on cotton in Australia', *Australasian Plant Pathology,* vol. 19, no. 1, pp. 24-25.

Allen, S.J., Nehl, D.B. & Moore, N. 'Appendix 1, Variety Resistance Rankings', in *Integrated Disease Management Manual*, Australian Cotton Cooperative Research Centre

Allen, S.J., Nehl, D.B. & Moore, N. 2008, Integrated Disease Management: Australian Cotton Cooperative Research Centre.

Barnett, S.J., Roget, D.K. & Ryder, M.H. 2006, 'Suppression of Rhizoctonia solani AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and *Microbacteria*', *Australian Journal of Soil Research,* vol. 44, no. 4, pp. 331-342.

Bashan, Y. & de-Bashan, L.E. 2002, 'Protection of Tomato Seedlings against Infection by Pseudomonas syringae pv. Tomato by Using the Plant Growth-Promoting Bacterium *Azospirillum brasilense*', *Applied and Environmental Microbiology,* vol. 68, no. 6, pp. 2637-2643.

BFA 2008, (updated 22/7), *New research reveals over 80% growth in organic farm gate sales since 2004 despite widespread drought*, Biological Farmers of Australia Retrieved from http://www.bfa.com.au/index.asp?Sec_ID=259

Broekaert, W.F., Terras, F.R. & Cammue, B.P.A. 2000, 'Induced and preformed antimicrobial proteins', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Fraser & L.C. Van Loon (eds), Kluwer Academic Publishers, Dordrecht

Candole, B.L. & Rothrock, C.S. 1998, 'Using marked strains to assess the effect of hairy vetch amendment on the inoculum densities of *Thielaviopsis basicola, Pythium ultimum* and *Rhizoctonia solani*', *Soil Biol. Biochem.,* vol. 30, no. 4, pp. 443-448.

Christou, T. 1962, 'Penetration and host-parasite relationships of *Thielaviopsis basicola* on the bean plant', *Phytopathology,* vol. 52, pp. 194-198.

Climate Averages, 2009, Bureau of Meteorology. Retrieved 27/01 2009 from http://www.bom.gov.au/climate/averages/

Colson-Hanks, E.S. & Deverall, B.J. 2000, 'Effect of 2,6-dichloroisonicotinic acid, its formulation materials and benzothiadiazole on systemic resistance to alternaria leaf spot in cotton ', *Plant Pathology,* vol. 49, pp. 171-178.

Conant, G.H. 1927, 'Histological studies of resistance in tobacco to *Thielavia basicola*', *American Journal of Botany,* vol. 14, no. 8, pp. 457-480.

Cotton Australia. 2008, 'Australian Cotton History', in *Fact Sheet Book*, Cotton Australia, Mascot, NSW

Cotton Research and Development Corporation Annual Report 2004. 2005. Narrabri: Cotton Research and Development Corporation.

Cotton-Australia 2009, *Facts and Figures: General; Cotton in Australia*. Retrieved from http://www.cottonaustralia.com.au/facts/factsandfigures.aspx?id=18

Current Drought Situation, 2008. Retrieved 16/1 2008 from http://www.dpi.nsw.gov.au/agriculture/emergency/drought/situation/drought-maps/drought-maps

Delevecchio, V.G., Corbaz, R. & Turian, G. 1969, 'An ultrastructural study of the hyphae, endoconidida and chlamydospores of *Thielaviopsis basicola*', *Journal of General Microbiology,* vol. 58, pp. 23-27.

Disease Ranks, 2008, Cotton Seed Distributors. Retrieved from http://www.csd.net.au/page/show/21091/

Edreva, A. 2004, 'A novel strategy for plant protection: Induced resistance', *Journal of Cell and Molecular Biology,* vol. 3, pp. 61-69.

El-Wakiel, N.E. & El-Sebai, T.N. 2008, 'Role of biofertilizer on faba bean growth, yield, and its effect on bean aphid and the associated predators ', *Archives of Phytopathology and Plant Protection*

Francis, J. 2006, 'GM Crops, the Precautionary Principle and Canola: Do the Exceptions Prove the Rule? ' *Australian Political Studies Association Conference*

Fukui, R., Fukui, H. & Alvarez, A.M. 1999, 'Suppression of Bacterial Blight by a Bacterial Community Isolated from the Guttation Fluids of Anthuriums', *Applied and Environmental Microbiology,* vol. 65, no. 3, pp. 1020-1028.

Fulchieri, M. & Frioni, L. 1994, 'Azospirillum inoculation on maize (Zea mays): effect on yield in a field experiment in central argentina', *Soil Biology and Biochemistry,* vol. 26, no. 7, pp. 921-923.

Gao, A., Hakimi, S.M., Mittanck, C.A., Wu, Y., Woerner, B.M., Stark, D.M., Shah, D.M., Liang, J. & Rommens, C.M.T. 2000, 'Fungal pathogen protection in potato by expression of a plant defensin peptide', *Nature Biotechnology,* vol. 18, pp. 1307-1310.

Gayed, S.K. 1969, 'The relation between tobacco leaf and root necrosis induced by *Thielaviopsis basicola* resistance to black root rot', *Phytopathology,* vol. 59, pp. 1596-1600.

Hake, K., DeVay, J., Kerby, P., Garber, R. & Chrisco, L. 1985, 'Cultural control of cotton black root rot', *Beltwide Cotton Production Research Conference*, pp. 25-26.

Harrison, U.J. & Shew, H.D. 2001, 'Effects of soil pH and nitrogen fertility on the population dynamics of *Thielaviopsis basicola*', *Plant and Soil,* vol. 228, pp. 147-155.

Harwood, R.R. 1990, 'A history of sustainable agriculture', in *Sustainable agricultural systems*, C.A. Edwards, R. Lal, P. Madden, H.R. Miller & G. House (eds), CRC Press, pp. 3-19.

Hoitink, H.A.J. & Fahy, P.C. 1986, 'Basis for the control of soilborne plant pathogens with composts', *Annual Review of Phytopathology,* vol. 24, pp. 93-114.

Hood, M.E. & Shew, H.D. 1997, 'Initial cellular interactions between *Thielaviopsis basicola* and tobacco root hairs', *Phytopathology,* vol. 87, no. 3, pp. 228-235.

Huang, H.C. & Patrick, Z.A. 1971, 'Variability of *Thielaviopsis basicola* in culture', *Canadian Journal of Botany,* vol. 49, no. 6, pp. 1041-1047.

Inglis, D.A. & Cook, R.J. 1986, 'Persistence of Chlamydospores of *Fusarium culmonorum* in Wheat Field Soils of Eastern Washington', *Phytopathology,* vol. 76, no. 11, pp. 1205-1208.

International Cotton Advisory Committee. 2007, Cotton: World statistics: Secretariat of the International Cotton Advisory Committee.

Jabs, T. & Slusarenko, A.J. 2000, 'The hypersensitive response', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Fraser & L.C. Van Loon (eds), Kluwer Academic Publishers, Dordrecht

Jetiyanon, K. & Kloepper, J.W. 2002, 'Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases', *Biological Control,* vol. 24, pp. 285-291.

Johnson, J. 1916, 'Host plants of *Thielavia basicola*', *Journal of Agricultural Research,* vol. 7, no. 6, pp. 289-300.

King, C.J. & Presley, J.T. 1942, 'A root rot of cotton caused by *Thielaviopsis basicola*', *Phytopathology,* vol. 32, pp. 752-761.

Knudsen, I.M.B., Hockenhull, J., Funck Jensen, D., Gerhardson, B., Hokeberg, M., Tahvonen, R., Teperi, E., Sundheim, L. & Henriksen, B. 1997, 'Selection of biological control agents for controlling soil and seed-borne diseases in the field', *European Journal of Plant Pathology,* vol. 103, pp. 775-784.

Kristyanne, E.S., Kim, K.S. & Stewart, J.M. 1997, 'Magainin effects on the ultrastructure of five plant pathogens', *Mycologia,* vol. 89, no. 3, pp. 353-360.

Lamb, C. & Dixon, R.A. 1997, 'The oxidative burst in plant disease resistance', *Annual Review of Plant Physiology and Plant Molecular Biology,* vol. 48, no. 1, pp. 251-275.

Last, D.I. & Llewellyn, D.J. 1997, 'Antifungal proteins from seeds of Australian native plants and isolation of an antifungal peptide from *Atriplex nummularia*', *New Zealand Journal of Botany,* vol. 35, pp. 385-394.

Lazarovits, G., Tenuta, M. & Conn, K.L. 2001, 'Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops', *Australasian Plant Pathology,* vol. 30, no. 2, pp. 111-117.

Leeman, M., Pelt, J.A.v., Ouden, F.M.d., Heinsbroek, M., Bakker, P.A.H.M. & Schoippers, B. 1995, 'Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*', *Phytopathology,* vol. 85, no. 9, pp. 1021-1027.

Lindow, S.E. 1988, 'Lack of correlation of In vitro antibiosiswith antagonism if ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria', *Ecology and Epidemiology,* vol. 78, pp. 444-450.

Liu, L., Kloepper, J.W. & Tuzun, S. 1995, 'Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria', *Phytopathology,* vol. 85, no. 8, pp. 843-847.

Lopez, L., Morales, G., Wolff, M. & Lowenberger, C. 2003, 'Isolation and characterization of a novel insect defensin from Rhodnius prolixus, a vector of Chagas disease', *Insect Biochemistry and Molecular Biology,* vol. 33, pp. 439-447.

Lucas, G.B. 1955, 'The cardinal temperatures and pH response of *Thielaviopsis basicola*', *Mycologia,* vol. 47, pp. 793-798.

Mathre, D.E. & Ravenscroft, A.V. 1966, 'Physiology of germination of chlamydospores and endoconidia of *Thielaviopsis basicola*', *Phytopathology,* vol. 56, pp. 337-342.

Mathre, D.E., Ravenscroft, A.V. & Garber, R.H. 1966, 'The role of *Thielaviopsis basicola* as a primary cause of yield reduction in cotton in California', *Phytopathology,* vol. 56, pp. 1119-1212.

Mauk, P.A. & Hine, R.B. 1988, 'Infection, colonization of *Gossypium hirsutum* and *G. barbadense*, and development of black root rot caused by *Thielaviopsis basicola*', *Phytopathology,* vol. 78, no. 12, pp. 1662-1667.

Meyer, J.R. & Shew, H.D. 1991a, 'Development of black root rot in burley tobacco as influenced by inoculum density of *Thielaviopsis basicola*, host resistance, and soil chemistry', *Plant Disease,* vol. 75, pp. 601-605.

Meyer, J.R. & Shew, H.D. 1991b, 'Soils suppressive to black root rot of burley tobacco, caused by *Thielaviopsis basicola*', *Phytopathology,* vol. 81, no. 9, pp. 946-954.

Meyer, J.R., Shew, H.D. & Harrison, U.J. 1994, 'Inhibition of germination and growth of *Thielaviopsis basicola* by aluminum', *Phytopathology,* vol. 84, pp. 598-602.

Mims, C.W., Copes, W.E. & Richardson, E.A. 2000, 'Ultrastructure of the penetration and infection of pansy roots by *Thielaviopsis basicola*', *Phytopathology,* vol. 90, no. 8, pp. 843-850.

Moerschbacher, B. & Mendgen, K. 2000, 'Structural aspects of defense', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Frase & L.C. van Loon (eds), Kluwer Academic Publishers, Dordrecht

Mondal, A.H., Nehl, D.B. & Allen, S.J. 2004, 'First report of *Thielaviopsis basicola* on soybean in Australia', *Australasian Plant Pathology,* vol. 33, pp. 451-452.

Mondal, A.H., Nehl, D.B. & Allen, S.J. 2005, 'Acibenzolar-S-methyl induces systemic resistance in cotton against black root rot casued by *Thielaviopsis basicola*', *Australasian Plant Pathology,* vol. 34, pp. 499-507.

Mondal, A.H., Nehl, D.B. & Deverall, B.J. 2000, 'Induced resistance can protect cotton and legumes from black root rot', *10th Australian Cotton Conference*

Nag Raj, T.R. & Kendrick, B. 1975, *A monograph of Chalara and allied genera*, Wilfred Lanvier Press, Waterloo, Ontario, Canada.

National Standard for Organic and Bio-dynamic Produce, 2005. Retrieved from http://www.ofa.org.au/papers/2005%20Draft%20NATIONAL%20STANDARD.pdf

Nehl, D.B., Allen, S.J., Lonergan, P.A., McNamara, G., Swan, L. & Smith, L.J. 2007, *Cotton Pathology 2006-2007*. Retrieved from http://www.cottoncrc.org.au/content/Industry/Publications/Disease__Microbiology/Disease_Surveys/2006_-_2007.aspx

Nehl, D.B., Allen, S.J., Mondal, A.H. & Lonergan, P.A. 2004, 'Black root rot: a pandemic in Australian cotton', *Australasian Plant Pathology,* vol. 33, pp. 87-95.

Nehl, D.B., Mondal, A.H. & Allen, S.J. (eds). 2000, *Managing black root rot,* Brisbane, Australia.

Nguyen, H.T., Deaker, R., Kennedy, I.R. & Roughley, R.J. 2003, 'The Positive Yield Response of Field-Grown Rice to Inoculation with a Multi-Strain Biofertiliser in the Hanoi Area, Vietnam ', *Symbiosis,* vol. 35, no. 1-3, pp. 231-245.

Nustorova, M., Braikova, D., Gousterova, A., Vasileva-Tonkova, E. & Nedkov, P. 2006, 'Chemical, microbiological and plant analysis of soil fertilized with alkaline hydrolysate of sheep's wool waste', *World Journal of Microbiology and Biotechnology,* vol. 22, no. 4, pp. 383-390.

O'Brien, R.G. & Davis, R.D. 1994, 'Lettuce black root rot - a disease caused by *Chalara elegans* ', *Australasian Plant Pathology,* vol. 23, pp. 106-111.

OGTR 2006, *Decision on issuing a licence for application DIR 066/2006; commercial release of five herbicide tolerant and/or insect resistant GM cotton lines in northern Australia*. Retrieved from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir066-2006

OGTR 2008, (updated 22/08/2008), *Legislation; The Regulatory Scheme for Genetically Modified Organisms*. Retrieved 1/2/2009 from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dirclass-2

Okon, Y. & Labandera-Gonzalez, C.A. 1994, 'Agronomic applications of *Azospirillum*: An evaluation of 20 years of worldwide field inoculation', *Soil Biol. Biochem.,* vol. 26, no. 2, pp. 1591-1601.

Organic Exchange 2007 Annual Report, 2008. Retrieved from http://www.organicexchange.org/Documents/annual07.pdf

Owen, K.J., Green, C.D. & Deverall, B.J. 2002, 'A benzothiadiazole applied to foliage reduces development and egg deposition by *Meloidogyne* spp. in glasshouse-grown grapevine roots', *Australasian Plant Pathology,* vol. 31, pp. 47-53.

Oyarzun, P.J., Gerlagh, M. & Zadoks, J.C. 1998, 'Factors associated with soil receptivity to some fungal root rot pathogens of peas', *Applied Soil Ecology,* vol. 10, pp. 151-169.

Paclt, J. 1960, 'Correct Name for the Type of Thielavia Zopf', *Taxonomy,* vol. 9, no. 1, pp. 26-27.

Papavizas, G.C. & Adams, P.B. 1969, 'Survival of root-infecting fungi in soil XII. Germination and survival of endoconidia and chlamydospores of *Thielaviopsis basicola* in fallow soil and in soil adjacent to germinating bean seed', *Phytopathology,* vol. 59, pp. 371-378.

Papavizas, G.C. & Kovacs Jr, M.F. 1972, 'Stimulation of Spore Germination of *Thielaviopsis basicola* by Fatty Acids from Rhizosphere Soil', *Phytopathology,* vol. 62, pp. 688-694.

Paulin-Mahady, A.E., Harrington, T.C. & McNew, D. 2002, 'Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis* anamorphs associated with *Ceratocyctis*', *Mycologia,* vol. 94, no. 1, pp. 62-72.

Paulitz, T., Nowak-Thompson, B., Gamard, P., Tsang, E. & Loper, J. 2000, 'A novel antifungal furanone from *Pseudomonas aureofaciens*, a biocontrol agent of fungal plant pathogens', *Journal of Chemical Ecology,* vol. 26, no. 6, pp. 1515-1524.

Perlak, F.J., Oppenhuizen, M., Gustafson, K., Voth, R., Sivasupraniam, S., Heering, D., Carey, B., Ihrig, R.A. & Roberts, J.K. 2007, 'Development and commercial use of Bollgard cotton in the USA - early promises versus todays reality', *The Plant Journal,* vol. 27, no. 6, pp. 489-501.

Posthuma, G.A., Schapper, W.M.M., Sijtsma, L., Van Amerongen, A., Fant, F. & Borremans, F.A.M. 2000, *Plant Defensin Variants*. Retrieved 26/1/2009 from http://www.wipo.int/pctdb/en/wo.jsp?IA=GB2000002941&DISPLAY=STATUS

Postma, J., Geraats, B.P.J., Pastoor, R. & van Elsas, J.D. 2005, 'Characterization of the Microbial Community Involved in the Suppression of Pythium aphanidermatum in Cucumber Grown on Rockwool', *Phytopathology,* vol. 95, no. 7, p. 808.

Pray, C.E., Huang, J., Hu, R. & Rozelle, S. 2002, 'Five years of Bt cotton in China - the benefits continue', *The Plant Journal,* vol. 31, no. 4, pp. 423-430.

Punja, Z.K. 1993, 'Influence of culture conditions on mycelial growth and phialospore production and germination in *Chalara elegans*', *Canadian Journal of Botany,* vol. 71, pp. 447-456.

Punja, Z.K. & Sun, L. 1999, 'Morphological and molecular characterization of *Chalara elegans (Thielaviopsis basicola)*, cause of black root rot on diverse plant species', *Canadian Journal of Botany,* vol. 77, pp. 1801-1812.

Pyke, B.A. (ed). 2007, *The impact of high adoption of Bollgard®II cotton on pest management in Australia,* Lubbock, TX, USA.

Raaijmakers, J.M., Vlami, M. & de Souza, J.T. 2002, 'Antibiotic production by bacterial biocontrol agents', *Antonie van Leeuwenhoeck,* vol. 81, pp. 537-547.

Ramos Solano, B., Barriuso Maicas, J., Pereyra de la Iglesia, M.T., Domenech, J. & Gutierrez Manero, F.J. 2008, 'Systemic Disease Protection Elicited by Plant Growth Promoting Rhizobacteria Strains: Relationship Between Metabolic Responses, Systemic Disease Protection, and Biotic Elicitors', *Phytopathology,* vol. 98, no. 4, pp. 451-457.

Reddy, M.S., Hynes, R.K. & Lazarovits, G. 1994, 'Relationship between in vitro growth inhibition of pathogens and suppression of preemergence damping-off and postemergence root rot of white bean seedlings in the greenhouse by bacteria ', *Canadian Journal of Microbiology,* vol. 40, no. 2, pp. 113-119.

Riggs, W. & Mims, C.W. 2000, 'Ultrastructure of chlamydospore development in the plant pathogenic fungus *Thielaviopsis basicola*', *Mycologia,* vol. 92, pp. 123-129.

Rothrock, C.S. 1992, 'Influence of Soil Temperature, Water, and Texture on *Thielaviopsis basicola* and Black Root Rot of Cotton', *Phytopathology,* vol. 82, no. 10, pp. 1202-1206.

Rothrock, C.S., Kirkpatrick, T.L., Frans, R.E. & Scott, H.D. 1995, 'The influence of winter legume cover crops on soilborne plant pathogens and cotton seedling diseases', *Plant Disease,* vol. 79, pp. 167-171.

Selitrennikoff, C.P. 2001, 'Antifungal proteins', *Applied and Environmental Microbiology,* vol. 67, no. 7, pp. 2883-2894.

Selvakumar, G., Joshi, P., Nazim, S., Mishra, P., Kundu, S. & Gupta, H. 2009, 'Exiguobacterium acetylicum strain 1P (MTCC 8707) a novel bacterial antagonist from the North Western Indian Himalayas', *World Journal of Microbiology and Biotechnology,* vol. 25, no. 1, pp. 131-137.

Sewell, G.W.F. & Wilson, J.F. 1966, 'Verticillium wilt of the hop: the survival of *V. albo-atrum* in soil', *Annals of Applied Biology,* vol. 58, no. 2, pp. 241-249.

Siddiqui, I.A. & Shaukat, S.S. 2002, 'Mixtures of plant disease suppressive bacteria enhance biological control of multiple tomato pathogens', *Biology and Fertility of Soils,* vol. 36, pp. 260-268.

Stockwell, V.O., Johnson, K.B., Sugar, D. & Loper, J.E. 2002, 'Antibiosis Contributes to Biological Control of Fire Blight by *Pantoea agglomerans* Strain Eh252 in Orchards', *Phytopathology,* vol. 92, no. 11, pp. 1202-1209.

Stover, R.H. 1950, 'The black rootrot disease of tobacco - Physiological specialization of *Thielaviopsis basicola* on *Nicotiana tabacum*', *Canadian Journal of Research,* vol. 28, pp. 726-738.

Stutz, E., Kahr, G. & Defago, G. 1989, 'Clays involved in suppression of tobacco black root rot by a strain of *Pseudomonas florescens*', *Soil Biology and Biochemistry,* vol. 20, no. 3, pp. 361-366.

Stutz, E.W., Defago, G. & Kern, H. 1985, 'Naturally occuring fluorescent *Pseudomonads* involved in suppression of black root rot of tobacco', *Disease Control and Pest Management,* vol. 76, pp. 181-185.

Teixidó, N., Usall, J., Palou, L., Asensio, A., Nunes, C. & Viñas, I. 2001, 'Improving Control of Green and Blue Molds of Oranges by Combining *Pantoea agglomerans* (CPA-2) and Sodium Bicarbonate', *European Journal of Plant Pathology,* vol. 107, no. 7, pp. 685-694.

Terras, F.R., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., VanLeuven, F., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. 1995, 'Small cysteine-rich antifungal proteins from radish: their role in host defense', *The Plant Cell,* vol. 7, pp. 573-588.

Terras, F.R.G., Schoofs, H.M.E., De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. 1992, 'Analysis of Two Novel Classes of Plant Antifungal Proteins from Radish (*Raphanus sativus* L.) Seeds', *Journal of Biological Chemistry,* vol. 267, no. 22, pp. 15301-15309.

Tien, T.M., Gaskins, M.H. & Hubbell, D.H. 1979, 'Plant Growth Substances Produced by Azospirillum brasilense and Their Effect on the Growth of Pearl Millet (Pennisetum americanum L.)', *Applied and Environmental Microbiology,* vol. 37, no. 5, pp. 1016-1024.

Tsao, P.H. & Bricker, J.L. 1966, 'Chalmydospores of *Thielaviopsis basicola* as surviving propagules in natural soils', *Phytopathology,* vol. 56, pp. 1012-1014.

Van Loon, L.C. 2000, 'Systemic induced resistance', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Fraser & L.C. Van Loon (eds), Kluwer Academic Publishers, Dordrecht

Vilas Alves, A., De Samblanx, G., Terras, F., Cammue, B. & Broekaert, W. 1994, 'Expression of functional Raphanus sativus antifungal protein in yeast', *FEBS Letters,* vol. 348, pp. 228-232.

Whipps, J.M. 2001, 'Microbial interactions and biocontrol in the rhizosphere', *Journal of Experimental Botany,* vol. 52, pp. 487-511.

Wilkinson, C.A., Rufty, R.C. & Shew, H.D. 1991, 'Inheritance of partial resistance to black root rot in burley tobacco', *Plant Disease,* vol. 75, no. 9, pp. 889-892.

Yarwood, C.E. 1946, 'Isolation of *Thielaviopsis basicola* from soil by means of carrot discs', *Mycologia,* vol. 38, pp. 346-348.

Yarwood, C.E. 1981, 'The occurrence of *Chalara elegans*', *Mycologia,* vol. 75, pp. 524-530.

2 Suppression of *Thielaviopsis basicola* growth by three soil bacteria, *Pantoea agglommerans*, *Exiguobacterium acetylicum* and *Microbacterium* sp.

2.1. Abstract

Combinations of three soil bacteria, *Pantoea agglommerans*, *Exiguobacterium acetylicum* and *Microbacterium* sp. (PEM) have previously been shown to inhibit infection of wheat by *Rhizoctonia solani* AG8 in soil tests. In this study they were found to suppress growth of another soil-borne pathogen, *Thielaviopsis basicola* towards cotton plants. In a series of *invitro* directional growth tests, growth of *T. basicola* was suppressed in the presence but not in the absence of PEM. Any combination of these species was effective in suppressing directional fungal growth towards cotton seedlings but individual species were not. In addition, combinations of individual species with a non-biocontrol species (*E. coli*) effectively suppressed fungal directional growth, indicating the effect was due to a non-specific interaction. Even though the plant was shown to be a factor in the suppression, the effect was not due to an active host plant response and was still present when a cotton root extract was used as a fungal attractant instead of intact plants.

2.2. Introduction

Black root rot, caused by *T. basicola*, has become a significant fungal disease of cotton in Australia over the last 20 years (Allen 1990; Nehl et al. 2004) and there is currently no effective treatment. When used as biological amendments, soil borne micro-organisms may be able to provide an answer to this, now widespread, disease.

A range of soil borne bacteria have been used to help protect plants from fungal, bacterial and viral pathogens. Species of *Pseudomonas*, *Bacillus* and *Serratia* have all been shown to inhibit plant diseases including fusarium wilt, bacterial leaf spot, cucumber mosaic virus and *Rhizoctonia* induced damping off (Jetiyanon & Kloepper 2002; Leeman et al. 1995; Liu et al. 1995).

In some cases, a mixture of multiple strains or species proved to be more effective than an individual strain (Jetiyanon & Kloepper 2002; Siddiqui & Shaukat 2002). A group of three species, *Pantoea agglomerans*, *Exiguobacterium acetylicum* and *Microbacterium* sp. (PEM) were isolated from soil that was suppressing infection of wheat by *Rhizoctonia solani* AG-8 (Barnett et al. 2006). The individual species did not reduce disease levels, but all three applied together led to significant disease reductions. The strain of *T. basicola* (BRIP40192) used in this study is a cotton isolate (Jan Dean, Q DPI&F) shown to strongly grow towards cotton seeds and seedlings *invitro* (Pereg & Al-Jaiidi, unpublished data) and to infect cotton roots in soil assays (Moulynox & Pereg, unpublished data). Preliminary studies in our lab indicated that PEM could suppress black root rot of cotton (Moulynox and Pereg, unpublished data) and that *T. basicola* exhibits significant directional growth towards host plants (Pereg & Al-Jaiidi, unpublished data).

Previous research by Barnett et al. (2006) showed that although there was no direct inhibition of fungal growth by PEM in vivo and no reduced fungal load in the soil in the presence of PEM, the presence of the three strains together in the soil could be used as an effective biological control measure to suppress disease caused by *Rhizoctonia solani* AG-8 in wheat growing systems. Therefore, the influence of the bacteria on fungal growth was tested in the presence of the plant to see whether PEM would interfere with directional growth of *T. basicola* towards host plants in vivo. These experiments were performed using a method previously developed in our lab to test directional growth of the fungus towards cotton and other plants (Pereg et al., unpublished).

The aim of the research reported here was to investigate the effect of a combination of bacteria on the growth of *T. basicola* in the presence of cotton (*Gossypium hirsutum*) roots and extracts.

2.3. Materials and methods

Growth of bacterial and fungal species

PEM (Barnett et al. 2006) and *E. coli* (DH5α) were all grown in Leuria Broth (LB) (10g/L Bacto tryptone, 5g/L NaCl, 5g/L Yeast extract) for 16-20 hours at 30°C/160 RPM before being used directly from culture. Where combinations of bacteria have been used, equal volumes from each overnight culture were mixed immediately before use.

T. basicola (BRIP40192) cultures were grown on half strength Potato Dextrose Agar (½ PDA medium; 18.5g/L Bacto PDA powder and15g/L Difco agar) at 25°C for 4-6 days and kept refrigerated at 4°C until use. Endoconidial spore suspensions were prepared by scraping the entire mycelial mat from an agar culture plate into several millilitres of sterile Milli-Q water. This suspension was then vortexed vigorously for 1 minute before filtering through two layers of Miracloth (Calbiochem) that had been soaked in 70% ethanol and dried under UV light to exclude hyphal fragments and chlamydospore chains. Spore concentration was determined by direct count using a haemocytometer. The suspension was then diluted as required using sterile Milli-Q water.

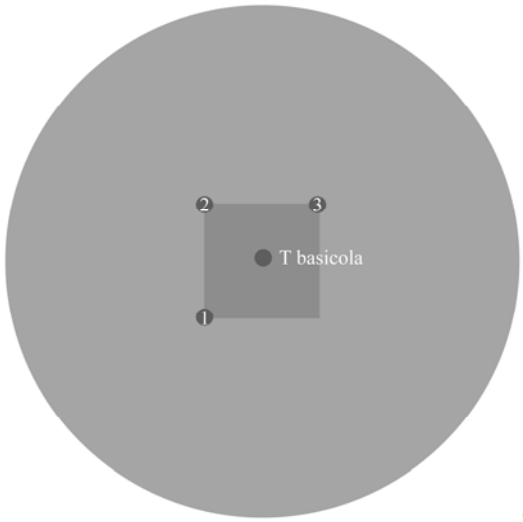
Cotton seeds

Cotton seeds (Sicot 189BR) were surface sterilised by soaking in 70% ethanol for 1 minute and 2% bleach for 5 minutes with gentle shaking. Seeds were then rinsed three times using sterile distilled H_2O (sdH_2O) then germinated on Yeast Mannitol Agar (YMA) plates (1g/L yeast extract, 10g/L mannitol, 0.5g/L K_2HPO_4, 0.2g/L $MgSO_4.7H_2O$, 15g/L agar) at 25°C in the dark for at least two days to check for contamination. Any seeds showing signs of contamination after the incubation period were discarded.

Direct invitro fungal hyphal growth inhibition testing

To determine if any one of the three bacterial species (PEM) were producing an anti-fungal agent that would be effective against *T. basicola*, individual bacterial species and *T. basicola* were stab inoculated 20mm apart onto Bacterial Fungal Growth Agar (BFGA) plates containing 2.2% agar, 18.5g/L Bacto PDA medium (½ strength), 5g/L Difco Yeast Extract, 15g/L Difco agar. Plates were then incubated at 25°C for 4-6 days.

To further examine the ability of the PEM bacteria to inhibit fungal hyphal growth invitro, plates with all three bacterial species were prepared (Figure 4a). *T. basicola* was stab inoculated at the centre of a BFGA plate and each of *Pantoea* (P), *Exiguobacterium* (E) and *Microbacterium* (M) were stab inoculated at equidistant points, 14mm from the centre. Plates were incubated at 25°C for 4-6 days. Table 1 shows the combinations of bacteria tested and number of replicates for each treatment.



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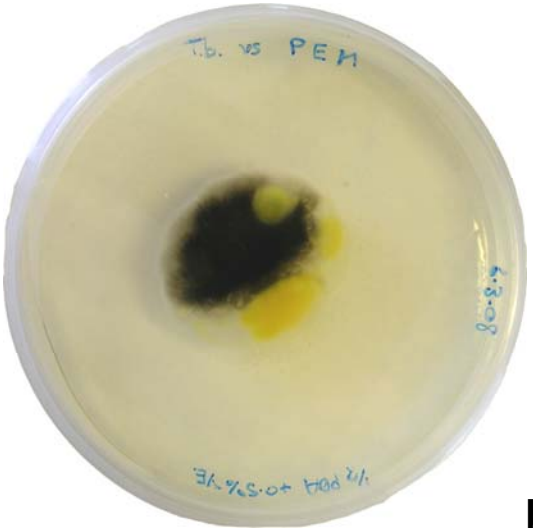

b

Figure 4 PEM interactions with *T. basicola*

a: Arrangement of inoculation sites for direct inhibition tests between the PEM bacteria and *T. basicola*.

b: The image shows a photo of a test plate after eight days, with *Pantoea* at position 1, *Exiguobacterium* at position 2 and *Microbacterium* at position 3. No zones of inhibition are visible.

Table 1: Combinations of PEM used to test growth of *T. basicola* towards cotton roots

Treatment	Source of stimulus for *T. basicola*	Number of plates per replicate	Number of experimental replicates
PEM	Seeds	5	3
P	Seeds	5	3
E	Seeds	5	3
M	Seeds	5	3
PE	Seeds	5	3
PM	Seeds	5	3
EM	Seeds	5	3
PEM	Root Extract	5	3

All possible combinations of the three PEM species were tested. Cultures were grown overnight at 28°C with shaking in LB. Individual species were used directly from culture. For combination treatments, equal volumes of each culture were mixed immediately before use.

Table 2: Each of the three PEM species were tested in combination with E. coli.

Treatment	Source of stimulus for *T. basicola*	Number of plates per replicate	Number of experimental replicates
P, E. coli	Root extract	5	1
E, E. coli	Root extract	5	1
M, E. coli	Root extract	5	1
E. coli	Root extract	5	1

Testing fungal growth inhibition by PEM in the presence of plants

The effect of the PEM bacteria on the germination of fungal spores and growth of hyphae towards cotton seeds was tested on 100mm square plates, containing 1.2% noble agar (12g/L Difco noble agar in Milli-Q water). An aliquot of 70μl of bacterial cultures (treatment) was applied to one strip of sterile filter paper (Whatman No 1

chromatography paper, 65mmx10mm) and 70μl of sterile dH_2O was applied as control to a second strip of sterile filter paper. The two strips were placed 15mm on either side of an identical strip containing approximately $3.5x10^4$ *T. basicola* endoconidia, which was placed at the centre of the plate. Four surface-sterilised cotton seeds (Sicot 189BR) were allowed to germinate on yeast mannitol plates at 25°C for 2 days until roots were between 5-10mm long. Two germinated seeds were placed 5mm away from each of the control and treatment strips, thus 20mm from the edge of the central strip containing the fungal spores. Growing cotton seedlings were removed and replaced on days two and four with fresh germinated seeds. Growth of fungal hyphae towards the germinating seeds on each side was measured from the edge of the central strip to the front of the growing hyphae after six days. Data was analysed using a one way ANOVA to determine the significance of any apparent difference between growth on the control side and growth on the bacterial treatment side.

Fungal growth inhibition by pairs of *P. agglomerans*, *E. acetylicum* and *Microbacterium* (P+E, P+M, E+M as treatments) and by each of P, E and M in combination with a strain of E. coli (DH5α) (P+DH5α, E+DH5α, M+DH5α as treatments), was examined using the fungal hyphal growth inhibition testing method described above, with root extract as the fungal attractant as described below. A sterile paper strip (65mmx5mm) was placed next to the treatment strips in place of the seeds and 25ul of root extract was applied. Growth of fungal hyphae towards the root extract on each side was measured from the edge of the central strip to the front of the growing hyphae six days after inoculation. The use of root extract both eliminates a variable (seedling growth) and is easier to use, as seeds do not need to be changed during the experiment, reducing the risk of contamination. Additionally, replacing roots with root extract eliminates the possibility of the plant reacting to fungal exudates and changing its metabolism. As a result, root extract was used as the stimulatory source instead of germinated seeds in all further experiments.

Testing for a host response of cotton induced by PEM

To test whether seedlings were actively producing anti-fungal substances in the presence of PEM, growing seedlings were replaced with seedling root extracts in fungal growth inhibition tests. Root extract was obtained by surface sterilizing cotton seeds, germinating on YMA plates for 3 days at 25°C, then harvesting the roots and crushing using a mortar and pestle with a small quantity of sterile dH_2O (approximately 1ml per 20 roots). An aliquot of 25μl of the root extract was applied to a paper strip (65mm x 5mm), which was placed 25mm from the edge of the central strip containing the fungal spores. Bacterial treatments and dH_2O controls were as described above (Section 2.3).

2.4. Results

Direct invitro fungal hyphal growth inhibition testing

When grown on BFGA plates in the absence of plant material, colonies from individual bacterial species (P, E or M) were observed to make contact with *T. basicola* colonies after four to six days growth. After 2 to 3 days further incubation, the *T. basicola* colonies surrounded the bacterial colonies. Furthermore, when studying all three bacterial species (PEM) inoculated on one plate in the presence of *T. basicola*, to test the possibility that an interaction between the bacterial species would induce production of antifungal agents, *T. basicola* colonies were round and had grown equally towards all three bacterial colonies after four to six days incubation. Further incubation saw the fungal colony continue to grow around and past the bacterial colonies (Figure 4b).

Testing fungal growth inhibition by PEM in the presence of plants

Figure 5 shows that fungal hyphal growth towards cotton seedlings on the side where bacteria had been applied was greatly and significantly reduced ($p<0.01$) when all three PEM species were applied together. The treatments using individual species and pair combinations are discussed below.

PEM suppress T. basicola *hyphal growth more in pairs or triplets*

In control experiments, significant fungal hyphal growth inhibition was also observed where each of the three PEM species was paired with *E. coli* (DH5a)(Figure 7),

($p<0.01$). The level of inhibition was similar to the level observed where pairs were made within the PEM species (Figure 5), with growth on the untreated side approximately doubling the growth on the treated side. *E. coli* (DH5a) did not significantly change the level of fungal hyphal growth. Figure 5 shows the effect of individual PEM species and pair combinations in inhibiting fungal hyphal growth. P induced a small significant increase in fungal hyphal growth ($p<0.05$). A small decrease in fungal hyphal growth was observed for the individual application of E ($p<0.01$). Application of M by itself led to no significant change in fungal hyphal growth. When tested in pairs, all three pair combinations (PE, PM, EM) induced similar levels of growth inhibition to all three species together.

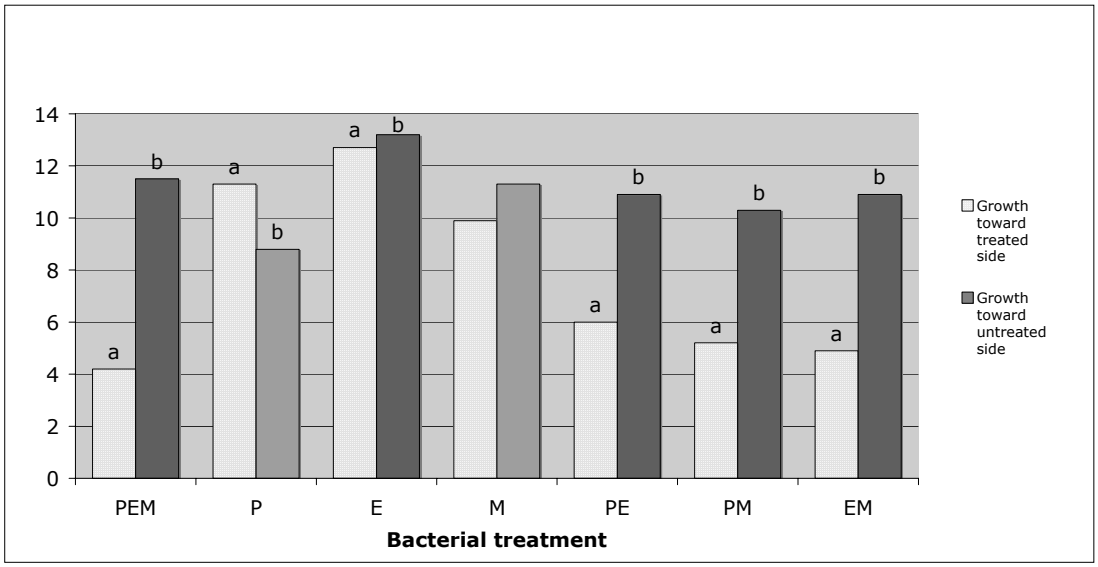

Figure 5: PEM effects on *T. basicola* directional growth towards cotton. The distance of fungal hyphal growth towards seedlings on the side of the plate treated with bacteria was compared to growth towards seedlings on the untreated side of the plate. Bacterial treatments were applied as combinations of all three strains (PEM) individual strains (P, E or M) or in pairs. (Data analysed using a one way ANOVA. a,b within a treatment indicates a significant difference ($p<0.01$). Data shown is the combined average data from three experimental replications of five plates each).

Testing for Host Response of cotton induced by PEM

Root extract was substituted for germinated cotton seeds in fungal growth inhibition tests to test for an induced host response. Figure 6 shows the fungal hyphal growth

both in the presence and absence of PEM bacterial treatment. There was no significant difference found between the fungal hyphal growth on the untreated side of the plate when root extract was used as the source of stimuli when compared to growth when germinated seeds were used. Additionally, the level of fungal hyphal growth on the side of the plate treated with PEM (and therefore any fungal hyphal growth inhibition), was not significantly different in the presence of seeds or root extract.

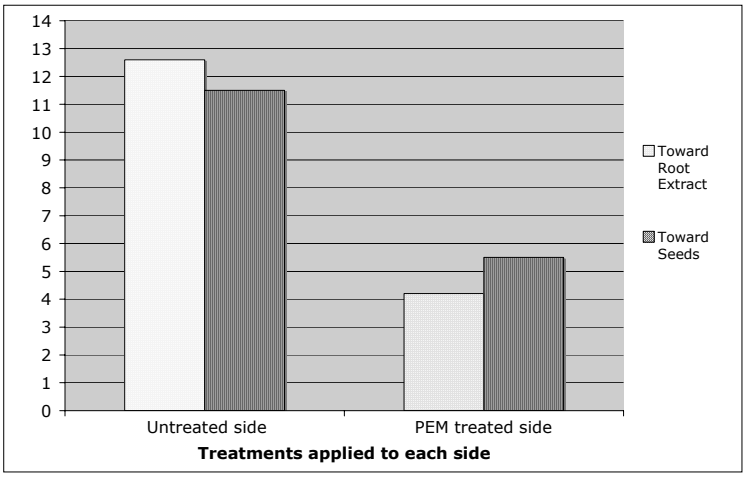

Figure 6: Fungal hyphal growth towards germinating cotton seeds or a crude root extract was compared either in the presence or absence of PEM bacterial treatments. There was no significant difference in fungal hyphal growth when using either cotton seeds or cotton root extracts.

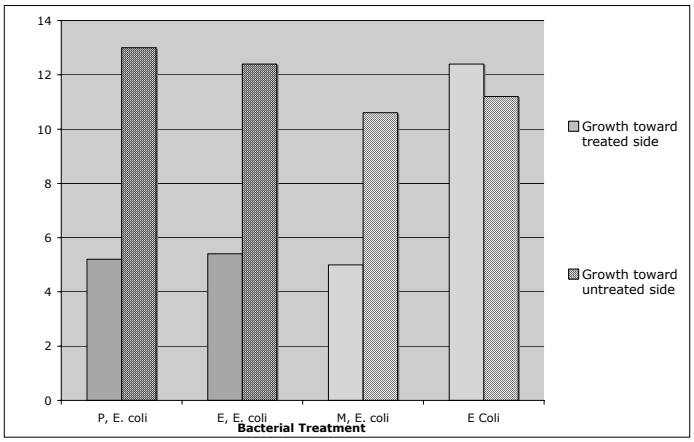

Figure 7: P, E and M were paired with *E. coli* (DH5α). Fungal growth towards root extract on the treated side of the plate was compared to growth towards root extract on the untreated side. Application of *E. coli* (DH5α) by itself led to a small increase in fungal growth (all results significant $p<0.01$).

2.5. Discussion

The use of soil bacterial species both individually and in combination to enhance crops by increasing growth rates or suppressing disease has been well established, however, the mechanisms are not always clear. For example, multiple strains of *Bacillus* applied to seeds can inhibit wilt disease in tomato caused by *Ralstonia solanacearum*, damping off in Pak choy due to *R. solani* and cucumber mosaic virus in cucumber seedlings even though the individual strains show no antibiosis toward the pathogens invitro (Jetiyanon & Kloepper 2002). Of the species used in this study, *P. agglomerans* has been shown to inhibit post-harvest *Penicillium* mold in oranges through an unknown mechanism (Teixidó et al. 2001) and reduce the incidence of fireblight in apples, partially via antibiosis (Stockwell et al. 2002). *Microbacterium* spp. have been implicated in suppression of *Pythium* damping off (Postma et al. 2005) and bacterial blight (Fukui et al. 1999) (both through undefined mechanisms) suggesting that at least two of the PEM species could inhibit a range of pathogens.

The initial directional growth testing in the presence of cotton roots or root extract, confirmed that *T. basicola* hyphal growth is suppressed in the presence of the PEM bacteria but did not provide any definitive evidence on the mechanism of the

suppression. Three hypotheses for the fungal growth inhibition in the presence of roots or root extract were considered; stimulation of the plant by the bacteria to actively produce a metabolite toxic to the *T. basicola*; an interaction between the species P, E and M that leads to bacterial production of a metabolite toxic to *T. basicola*; or the utilisation by the bacteria of compounds from the cotton roots involved in signalling or stimulating the fungal growth.

Production of an inhibitory metabolite by the plant under stimulation from the bacteria was considered as a source of the observed suppression, as exposure of plants to some bacterial species is known to induce host resistance against disease. Examples include the protection of radish against fusarium wilt by prior exposure to *Pseudomonas fluorescens* (Leeman et al. 1995), induction of resistance in cucumber to bacterial angular leaf spot by *Pseudomonas putida and Serratia marcescens* (Liu et al. 1995) or reduced damping off caused by *R. solani* when plants were exposed to certain Bacillus strains (Jetiyanon & Kloepper 2002). If a host response was occurring, it would be expected that it would require intact plants to respond to bacterial stimulation, for example by producing salicylic acid (Metraux 2001) or synthesis of new proteins (Chisholm et al. 2006; Van Loon 1997) that may be involved in a host response to a pathogen. When a crude cotton seed root extract was used as the stimulatory source instead of growing cotton seedlings, there was no significant change in the pattern of *T. basicola* hyphal growth in the absence of PEM bacterial treatment. This indicates that the root extract was equally as attractive to the fungi as germinated seeds. In addition, the amount of fungal growth suppression by PEM was also unchanged. These results eliminated the possibility that the fungal growth suppression by the bacteria was due to induction of an active host resistance response.

The individual PEM species had either a stimulatory effect or only a small suppressive effect on fungal hyphal growth while in any combination, a large and significant suppression of *T. basicola* growth was observed, confirming that in these tests an interaction is occurring between the species leading to suppression of *T. basicola* fungal hyphal growth. The interaction required to suppress *R. solani* infection of wheat soil appears to require all three species (Barnett et al. 2006) while the interaction that leads to suppression of *T. basicola* invitro only requires any one of the three species plus a competing species. Additionally, the effect observed in our

experiments is not an additive effect, with the level of suppression when combinations of species are used many times greater than the individual species.

As the PEM bacteria were only effective in suppressing fungal hyphal growth in pair or triplet combinations, the fungal hyphal growth inhibition is not simply an additive effect, with individual species providing very little or no suppression, and the plant has been excluded as the direct source of any anti-fungal metabolites, it can be concluded that the observed suppression is due to an interaction occurring between the bacterial species. The results of Barnett et al. (2006) imply that there is a specific interaction, as only the three species together reduced *R. solani* infection when tested in a soil system. The two experiments presented here using seed and root extract confirm an interaction, but do not show whether this fungal hyphal growth inhibition was due to a specific interaction within these three species or a more general competition interaction. Additionally, these experiments measured suppression of fungal hyphal growth, whereas Barnett et al. (2006) used infection of roots as a measure of disease suppression. The different observations could be as a result of dealing with two different fungal pathogens (*R. solani* and *T. basicola*), with a different mechanism is involved in the PEM bacteria suppressing each pathogen, and in comparing root infection in soil to hyphal growth towards roots.

Using *E. coli* (DH5α) in combination with any of the three PEM species (DH5α/P, DH5α/E or DH5α/M) produced a similar level of fungal hyphal growth inhibition as in the test using pairs of the PEM bacteria. This result suggested that all three of the PEM species are individually capable of inhibiting fungal hyphal growth given the right stimulation.

Much research has been focused on the importance of competition for limited nutrients among rhizosphere bacteria with several reviews covering the main areas including production of antibiotics (direct antagonism), (Raaijmakers et al. 2002) siderofores (competition for iron) or chitinases (lysing competing cells)(O'sullivan & O'gara 1992; Raaijmakers et al. 2002; Van Loon 1997; Whipps 1997, 2001). In the case of PEM species, it is likely that stimulation was competition for limited nutrients on the noble agar plates, however, the nature of the interaction was not further investigated. We were unable to test if addition of nutrients would reduce the level of fungal hyphal growth inhibition caused by competition between the bacterial species

using the agar plate method developed here, as addition of nutrients to the agar plates would have introduced a confounding factor when assessing the growth of the fungal hyphae towards the cotton roots. In initial experiments when developing the method (data not presented) nutrient agar was tested, however, the growth of the bacteria was too rapid and they colonised the entire plate within the 6 days required to measure the fungal hyphal growth, making it impossible to detect any fungal hyphal growth inhibition effects. Additionally, nutrient levels in bulk soil are relatively low but are significantly higher in the rhizosphere and competition between species is highly likely to occur (Rengel & Marschner 2005), making this system, using low nutrient agar and competition between species in the presence of roots or root extracts, more realistic and possibly more likely to detect effects that can be transferred to natural systems.

The initial invitro plate testing of the PEM bacteria for an inhibitory effect on *T. basicola* in the absence of plants was negative. This is in agreement with Barnett et al. (2006) who found no significant reduction in fungal load in PEM containing soil. These bacteria had already been tested in previous research and shown to have a significant inhibitory effect on another fungal pathogen, *R. solani* (Barnett et al. 2006). As some researchers have reported no relationship between invitro plate inhibition and inhibition in pot tests (Reddy et al. 1994) further testing of disease suppression by PEM in a more complex system was performed (Chapter 3). The later tests confirmed the inhibitory effect of the PEM bacteria in a controlled pot system, highlighting the importance of the system chosen to screen species for anti-fungal activity.

This study has clearly shown that the PEM bacteria can inhibit the directional growth of a cotton strain of *T. basicola* towards its host. The PEM species do not have any inhibitory effect on fungal hyphal growth individually, but competition between the bacterial species for limited nutrients may lead to a change in metabolism that then causes inhibition of fungal hyphal growth. Follow up work, examining molecules excreted from the PEM bacteria under different growth conditions will be required to identify whether there is an inhibitory substance or substances being excreted by the bacterial species, or if they are causing an attenuation of the signal that causes the directional growth. Additionally, this confirmation of suppressive activity can lead to

testing these species against *T. basicola* in the field in order to develop a treatment for a significant, widespread and currently untreatable disease in Australian cotton.

2.6. Acknowledgements

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2.7. References

Allen, S.J. 1990, '*Thielaviopsis basicola*, a new record on cotton in Australia', *Australasian Plant Pathology,* vol. 19, no. 1, pp. 24-25.

Barnett, S.J., Roget, D.K. & Ryder, M.H. 2006, 'Suppression of *Rhizoctonia solan*i AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and *Microbacteria*', *Australian Journal of Soil Research,* vol. 44, no. 4, pp. 331-342.

Chisholm, S.T., Coaker, G., Day, B. & Staskawicz, B.J. 2006, 'Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response', *Cell,* vol. 124, no. 4, pp. 803-814.

Fukui, R., Fukui, H. & Alvarez, A.M. 1999, 'Suppression of Bacterial Blight by a Bacterial Community Isolated from the Guttation Fluids of Anthuriums', *Applied and Environmental Microbiology,* vol. 65, no. 3, pp. 1020-1028.

Jetiyanon, K. & Kloepper, J.W. 2002, 'Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases', *Biological Control,* vol. 24, pp. 285-291.

Leeman, M., Pelt, J.A.v., Ouden, F.M.d., Heinsbroek, M., Bakker, P.A.H.M. & Schoippers, B. 1995, 'Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of Pseudomonas fluorescens', *Phytopathology,* vol. 85, no. 9, pp. 1021-1027.

Liu, L., Kloepper, J.W. & Tuzun, S. 1995, 'Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria', *Phytopathology,* vol. 85, no. 8, pp. 843-847.

Metraux, J. 2001, 'Systemic acquired resistance and salicylic acid: current state of knowledge', *European Journal of Plant Pathology,* vol. 107, pp. 13-18.

Nehl, D.B., Allen, S.J., Mondal, A.H. & Lonergan, P.A. 2004, 'Black root rot: a pandemic in Australian cotton', *Australasian Plant Pathology,* vol. 33, pp. 87-95.

O'Sullivan, D.J. & O'Gara, F. 1992, 'Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens', *Microbiology and Molecular Biology Reviews,* vol. 56, no. 4, pp. 662-676.

Postma, J., Geraats, B.P.J., Pastoor, R. & van Elsas, J.D. 2005, 'Characterization of the Microbial Community Involved in the Suppression of *Pythium aphanidermatum* in Cucumber Grown on Rockwool', *Phytopathology,* vol. 95, no. 7, p. 808.

Raaijmakers, J.M., Vlami, M. & de Souza, J.T. 2002, 'Antibiotic production by bacterial biocontrol agents', *Antonie van Leeuwenhoeck,* vol. 81, pp. 537-547.

Reddy, M.S., Hynes, R.K. & Lazarovits, G. 1994, 'Relationship between in vitro growth inhibition of pathogens and suppression of preemergence damping-off and postemergence root rot of white bean seedlings in the greenhouse by bacteria ', *Canadian Journal of Microbiology,* vol. 40, no. 2, pp. 113-119.

Rengel, Z. & Marschner, P. 2005, 'Nutrient availability and management in the rhizosphere: exploiting genotypic differences', *New Phytologist,* vol. 168, no. 2, pp. 305-312.

Siddiqui, I.A. & Shaukat, S.S. 2002, 'Mixtures of plant disease suppressive bacteria enhance biological control of multiple tomato pathogens', *Biology and Fertility of Soils,* vol. 36, pp. 260-268.

Stockwell, V.O., Johnson, K.B., Sugar, D. & Loper, J.E. 2002, 'Antibiosis Contributes to Biological Control of Fire Blight by *Pantoea agglomerans* Strain Eh252 in Orchards', *Phytopathology,* vol. 92, no. 11, pp. 1202-1209.

Teixidó, N., Usall, J., Palou, L., Asensio, A., Nunes, C. & Viñas, I. 2001, 'Improving Control of Green and Blue Molds of Oranges by Combining *Pantoea Agglomerans* (CPA-2) and Sodium Bicarbonate', *European Journal of Plant Pathology,* vol. 107, no. 7, pp. 685-694.

Van Loon, L.C. 1997, 'Induced resistance in plants and the role of pathogenesis-related proteins', *European Journal of Plant Pathology,* vol. 103, no. 9, pp. 753-765.

Whipps, J.M. 1997, 'Biological Control of Soil-borne Plant Pathogens', in *Advances in Botanical Research*, Academic Press, Birmingham, 26, pp. 1-134.

Whipps, J.M. 2001, 'Microbial interactions and biocontrol in the rhizosphere', *Journal of Experimental Botany,* vol. 52, pp. 487-511.

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	Author's Name (please print clearly)	**% of contribution**
Candidate	Jason Moulynox	70
Other Authors	Dr Lily Pereg	30

All experimental work is the work of the candidate. The Principal Supervisor developed the directional growth method on which the experiments are based and assisted with preparation of the manuscript.

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3 Four bacterial strains, *Azospirillum brasilense*, *Pantoea agglomerans*, *Exiguobacterium acetylicum* and *Microbacterium* sp inhibit black root rot symptoms in cotton in pot trials but not in the field

3.1. Abstract

Black root rot is a significant fungal pathogen in Australian cotton with no suitable treatment. *Azospirillim brasilense* has been shown to promote growth and suppress disease in other crops. *Pantoea agglomerans*, *Exiguobacterium acetylicum* and *Microbacterium* sp (PEM) have been shown to suppress multiple fungal pathogens and to inhibit growth of *Thielaviopsis basicola* invitro. In pot trials, application of *A. brasilense* or PEM to cotton seeds planted in soil infested with *T. basicola* significantly reduced black root rot symptoms by 29% and 37% respectively. These species were then applied as a planting treatment in field trials at multiple sites in NSW, Australia. None of the bacterial treatments significantly reduce disease symptoms under field conditions.

3.2. Introduction

Black root rot, caused by *Thielaviopsis basicola* is a significant fungal disease of cotton crops in most Australian cotton growing regions (Nehl et al. 2004). The disease causes blackening of the tap root and reduced plant vigour and can lead to significant yield losses. Disease surveys first detected affected cotton in Australia near Moree in northern NSW in 1989 (Allen 1990). Since then, regular disease surveys have followed its spread and in 2003, the disease was detected on 97% of surveyed cotton farms in northern NSW (Nehl et al. 2004).

Once it invades root tissue, *T. basicola* produces chlamydospores in large numbers that can remain dormant in the soil for several years until a suitable host crop is planted. Recent studies in Australian cotton growing regions have shown that the single most significant factor in the severity of disease in a given season is the number of host cotton crops grown previously (Nehl et al. 2004). Rotation with non-host crops such as wheat has little effect.

There are currently no commercial varieties of cotton in Australia that have any significant resistance to black root rot (pers. comm. Stephen Allen, Cotton Seed Distributors). There are also no treatments available to growers (Allen et al. 2008). It may be possible to reduce the impact of this disease by supplementing soil at planting with bacterial species that can reduce the severity of the disease symptoms.

Many bacterial species have been suggested as biocontrol agents for a range of crop diseases. Species of *Pseudomonas*, *Bacillus* and *Serratia* have all been shown to inhibit plant diseases including fusarium wilt, bacterial leaf spot and *Rhizoctonia* induced damping off (Jetiyanon & Kloepper 2002; Leeman et al. 1995; Liu et al. 1995). Selection of species that are effective under the highly controlled conditions of a laboratory provides no guarantee that they will be effective when transferred into in natural systems (Lindow 1988a; Reddy et al. 1994). For example Reddy et al. (1994) screened 120 bacteria for their ability to suppress *Pythium ultimum*, *Rhizoctonia solani* and *Fusarium solani* f.sp. phaseoli. A number of strains effectively suppressed disease in greenhouse trials, but these strains did not correlate with tests on solid culture media.

A. brasilense has been used successfully as a growth promoting species in many different crops (Okon & Labandera-Gonzalez 1994). As black root rot is a seedling disease and most cotton plants usually outgrow *T. basicola* infections (Allen et al. 2008), a plant growth promoting bacterial species may assist the plants to do this more quickly.

Pantoea agglomerans, *Exiguobacterium acetylicum* and *Microbacterium* sp. (PEM) suppress infection of wheat by *Rhizoctonia solani* AG-8 and members of the group, such as P, were shown to have plant growth promoting potential (Barnett et al. 2006). The PEM bacteria have been isolated from disease suppressive soils of wheat in South Australia and shown to be effective against *R. solani* AG8 under laboratory conditions in soil systems (Barnett et al. 2006). They also inhibit the directional growth of *T. basicola* towards cotton plants invitro (Moulynox, Chapter 2).

The aim of this study was to test *A. brasilense* and the PEM bacteria, first under laboratory conditions and then under field conditions, as bio-inoculants to reduce black root rot symptoms on working cotton farms in northern NSW, Australia.

3.3. Materials and methods

Microbial strains and media

For all pot trials, cultures of *P. agglomerans*, *E. acetylicum* and *Microbacterium* (Barnett et al. 2006) were grown in 3 to 5ml of Luria-Bertani (LB) in McCartney bottles for 16-20 hours at 30°C/160 RPM. Cultures of *A. brasilense* Sp7 (Tarrand et al. 1978; Pereg-Gerk 2004) were grown in 3-5ml of Nutrient Broth (NB, Difco) for 48 hours at 30°C/160RPM. Cultures of *A. brasilense* were observed microscopically before use to confirm the cells were still in a vegetative state. Cell numbers were not measured for each experiment, however measurements performed during prior experiments determined these growth conditions to produce between 10^6 of 10^7 cells/ml. Where combinations of bacteria have been used, equal volumes from each culture were mixed immediately before use.

For field trials, 10 ml starter cultures were first grown overnight in LB (PEM) (30°C/160 RPM) or NB (*A. brasilense*). Cultures for use in field inoculation were prepared by adding the 10 ml starter cultures to 500ml of fresh LB in 1L flasks and incubating for 16-24 hours (PEM) or 40-48 hours (*A. brasilense)* at approximately 25°C with shaking.

T. basicola (BRIP40192) (Jan Dean, Q DPI&F) cultures were grown on half strength Potato Dextrose Agar (½ PDA medium; 18.5g/L Bacto PDA powder and15g/L Difco agar) at 25°C for 4-6 days and kept refrigerated at 4°C until use.

Disease suppression assays using pot trials

Preparation of soil and seeds

Soil, classified as an alkaline grey-brown clacking clay (Ward et al. 1999), was collected from an active cotton growing field at ACRI (Narrabri, NSW) and sterilised

by autoclaving three times with 24hr intervals. A sterile mixture of sand and soil (50:50w/w) was inoculated with *T. basicola* endoconidial spore suspensions, which were prepared according to the method described in section 2. The endoconidial suspension was added to the sterile sand:soil mix to give a final spore concentration of $5x10^3$ spores/g soil. Inoculated soil was placed into 85ml or 200ml pots to be used in disease suppression experiments.

Cotton seeds (Sicot 189BR) were surface sterilised using $HgCl_2$ as follows: 1min Tween 20 detergent (1ml/L), rinse with sterile dH_20, 75 seconds 0.5% $HgCl_2$, rinse five times sterile dH_2O). Disease suppression experiments were conducted using pre-germinated seedlings as well as using seeds. For germination, seeds were placed on Yeast Mannitol Agar (YMA; 1g/L yeast extract, 10g/L mannitol, 0.5g/L K_2HPO_4, 0.2g/L $MgSO_4.7H_2O$, 15g/L agar) and incubated at 25°C in the dark for 2 days. Contaminated seeds were excluded.

Pot trials using cotton seedlings pre-inoculated with bacteria

A. brasilense Sp7 was shown to be capable of colonising the surface of cotton roots in a hydroponic system (Pereg Gerk, unpublished). Germinated cotton seedlings were inoculated with *A. brasilense* Sp7 (see below) prior to exposure to *T basicola* to ensure that the roots were well colonised.

Germinated cotton seeds with emerging roots approximately 5-10mm long were transferred to 20ml glass test tubes, each containing a folded paper support (Whatman #1 chromatography paper) placed in approximately 5ml of hydroponic solution (0.13M $CaCl_2$, 0.1M KH_2PO_4, 0.053M K_2HPO_4, 0.1M $MgSO_4$, 0.009M Fe-EDTA, plus 1ml/L microelement solution 0.025M H_3BO_3, 0.0005M $CoCl_2$, 0.0056M $MnSO_4$, 0.00001M $(NH_4)_6Mo_7O_{24}$, 0.002M $ZnSO_4$, 0.00025M $NaSO_4$) such that the seed could sit on the support above the surface of the liquid with the germinated root pointing down and entering the liquid (Figure 8) and allowed to grow for a further 3 days. Plants were treated with aliquots of 250µl of a 48 hour old culture of *A. brasilense* Sp7 (still in vegetative state), grown in NB, which was added to the hydroponic solution. Seedling growth continued for another 5 days to allow colonisation of roots by the bacteria. Control plants did not have bacteria added. Seedlings were then planted into prepared 85ml pots containing *T. basicola*

inoculated soil and incubated on a 12/12 light dark cycle at 23/18°C. Ten seedlings pre-colonised with *A. brasilense* were examined for tap root lesions caused by *T. basicola* thirteen days post planting.

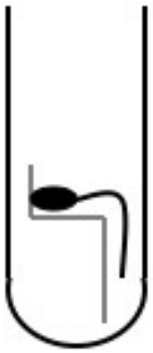


Figure 8: Cotton seedling resting on a paper support in a 20ml glass test tube. The germinating root is pointing down into 5-7ml of hydroponic solution. *A. brasilense* was added to the hydroponic solution in order to inoculate the growing roots prior to planting in pots.

Pot trials using non-germinated cotton seeds

To better simulate the situation in the field, pot trials were also conducted using non-germinated sterile cotton seeds. *T. basicola* inoculated soil in 200ml pots was supplemented with either 250µl of a 48 hr culture of *A. brasilense* Sp7 or 250µl of a 24hr culture of the PEM that had been grown individually then mixed in equal volumes immediately prior to use. All bacterial cultures were applied directly to the planting hole (approximately 20mm deep) along with the surface sterilised cotton seeds. Control pots were inoculated with 250µl of sterile dH2O, LB or NB instead of bacteria. Pots were incubated on a 12/12 light dark cycle at 23/18°C. A total of 15 seeds were planted for each treatment (one seed per pot). Seeds that did not germinate were excluded from assessment.

Assessment of disease severity in pot trials

In all pot trials, seedling tap-roots were scored for disease symptoms after 13 days of incubation by measuring the total length of tap root and the length of tap root exhibiting *T. basicola* lesions. A score was assigned on a scale of 0-10 representing

the proportion of tap root showing lesions where ten is equal to 100% blackening. This scoring method was consistent with current disease survey practices for black root rot in the field (Nehl et al. 2004).

Disease suppression assays in field trials

Field Trials

Field trial sites were selected in consultation with farm managers based on their history of black root rot. Due to operational restrictions (limited water availability and grower rotation plans) different fields were used each season. Five sites were used overall, three at ACRI, Narrabri, one near Wee Waa NSW and one near Trangie, NSW. Black root rot caused by *T. basicola* had either been detected in disease surveys or reported by the grower in the previous season at ACRI and Wee Waa. The Trangie site had been fallow for the previous three consecutive seasons so disease surveys were not completed in those years, however, disease surveys had detected black root rot at the Trangie site prior to the field being left fallow.

The 2005/06 trial was conducted at the Australian Cotton Research Institute (ACRI) approximately 15km north, north-west of Narrabri, NSW. The field had been under long term cotton/wheat rotation. In the 2006/07 season there were three sites. The first site was again at ACRI in a different section of the same field as in 2005/06. The second site was on a commercial cotton farm near Wee Waa, NSW, and had been planted to cotton followed by a winter fallow in the previous season. The third site was at a commercial farm near Trangie NSW. The field at Trangie had been fallow for three seasons prior to this trial due to limited water. The 2008/09 trial was again conducted at ACRI, in a different field to the 2005/06 and 2006/07 trials. This field was under continuous wheat cotton rotation. All sites were managed under a permanent bed, furrow irrigation system. Soil was either grey or grey-brown cracking clay at all sites (Ward et al. 1999).

Table 3: Field trial locations and recent paddock history

Location	2004/05	2005/06	2006/07	2007/08	2008/09
ACRI Site 1	Cotton/Wheat	*Cotton Trial*	NA	NA	NA
ACRI Site 2	Cotton/Wheat	Cotton/Wheat	*Cotton Trial*	NA	NA
ACRI Site 3	Cotton/Wheat	Cotton/Wheat	Cotton/Wheat	Cotton/Wheat	*Cotton Trial*
Wee Waa	Cotton/Fallow	Cotton/Fallow	*Cotton Trial*	NA	NA
Trangie	Fallow	Fallow	*Cotton Trial*	NA	NA

Cultures of PEM or *A. brasilense* in complete media (NB, 25°C/16-24hrs with shaking or LB, 25°C/40-48hrs with shaking, respectively) were applied directly to the planting furrow at a rate of approximately 250µl per seed using custom spray equipment attached to a standard 4 or 8 row planter. The spray nozzles were located behind the seed tube so that the seed fell into the planting furrow prior to spraying with the bacterial treatment. This application rate was similar to that applied in the invitro pot trials. Cotton cultivars were selected by the growers (Table 4) and seed was planted at 15 seeds/m. With the exception of the Trangie site, fields were all irrigated or had experienced significant rain prior to planting and so cotton was planted into a moist soil profile. The Trangie site was planted into a dry profile and was flood irrigated 16 days post-planting.

Table 4: Cotton cultivar planted at each trial site. Any seed treatments are also listed. None of these seed treatments are recommended for suppressing black root rot. Additionally, none of these varieties are recommended as being more resistant to black root rot.

Site	Cotton cultivar	Seed treatments
ACRI 2005/06	Sicot V3-BR	Dynasty
Wee Waa	Sicot 43BR	Dynasty
Trangie	Sicot 71BR	Dynasty/Peridiam
ACRI 2006/07	Sicot 71BR	Dynasty
ACRI 2008/09	Sicot 43BRF	Dynasty/Peridiam

Rows were standard one metre spacing and cotton was planted in every row. A buffer of untreated cotton at least 20m long was maintained at each end of the rows. Trials were laid out in blocks across four adjacent rows. Across one block, each treatment (*A. brasilense*, PEM or water control) was applied once. The treatment order was changed in each of three consecutive blocks so that each of the treatments was applied to three different rows. This pattern was then repeated giving a total of six blocks. A minimum of 20 linear metres was planted before each change. Where fields were not long enough to fit all six blocks in one set of rows, adjacent rows were used. The result was treatments arranged in a replicated split block design with a total of six replicates per treatment.

Current practice for cotton black root rot surveys in Australia is to assess for disease symptoms 4-6 weeks post planting (Nehl et al. 2004). In order to best cover the early season infection, seedlings in the trial were assessed twice for black root rot symptoms at 3 weeks and 5 weeks post-planting. At each assessment, a total of 60 plants from each treatment, representing ten plants from each of six replicates, were assessed for disease symptoms. Assessments were completed by selecting a transect across the rows and then removing the ten plants directly after the transect from the ground and assessing for disease severity. Disease severity was determined by measuring the length of the tap root that displayed the characteristic lesions and the total length of the root and assigning a score out of ten. As this assessment method is destructive, different transect were chosen for the two assessment dates. Random representative plants were examined microscopically to confirm the presence of *T. basicola* spores. Data was then analysed using one-way ANOVA to test for significant differences between treatments.

3.4. Results

A. brasilense *suppresses black root rot of cotton invitro*

Invitro soil assays showed that *A. brasilense* Sp7 suppressed black root rot of cotton seedlings in soil containing high numbers ($5x10^3$ spores/gram) of *T. basicola* spores. Disease levels in the *A. brasilense* treated plants invitro were similar for both cotton plants that were pre-colonised with bacteria in hydroponics system and plants that were treated with bacteria during seed planting (Figure 9). In both cases, statistical

analysis of the results using a one-way ANOVA showed that disease levels in the *A. brasilense*-treated cotton seedlings were significantly lower ($p<0.01$) than in the untreated control plants in soil containing *T. basicola*.

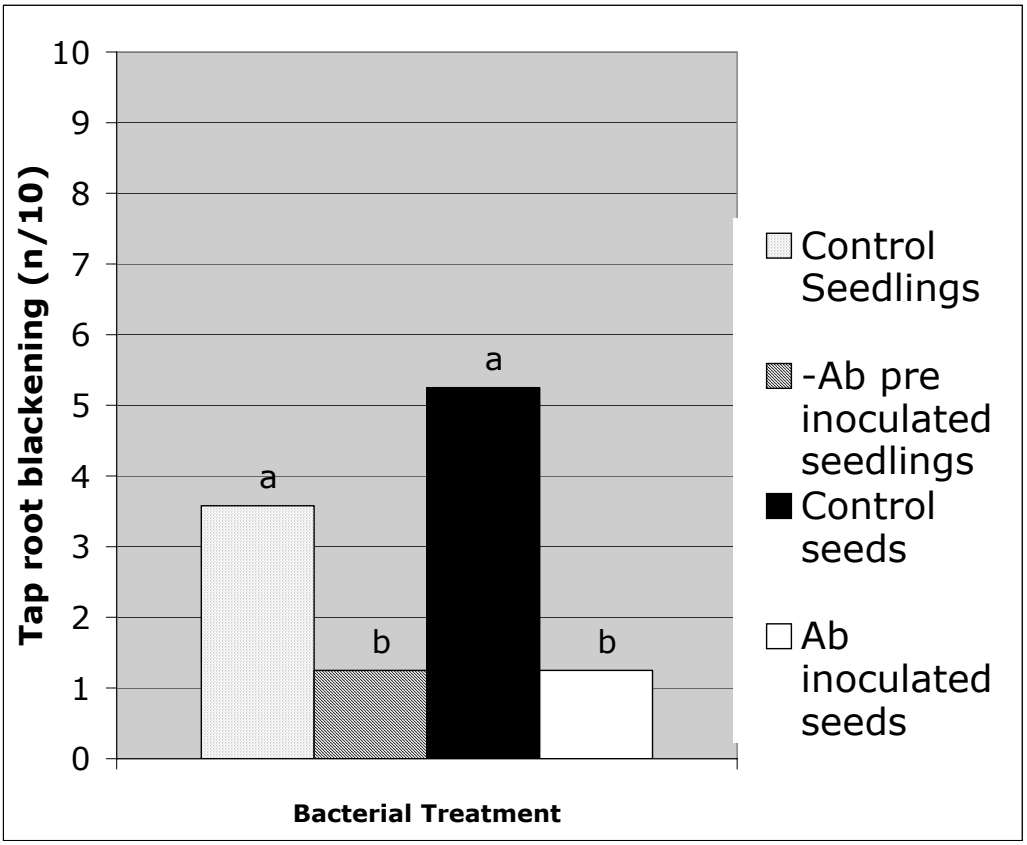


Figure 9: Cotton seeds treated at planting with *Azospirillum brasilense* (Ab) were compared with seedlings that had been pre-germinated and treated with A. brasilense prior to exposure to disease pressure by *T. basicola*. Control plants were no inoculated with bacteria (a,b within a treatment indicates significance at $p<0.01$, $n=10$)

In control plants, disease levels were lower in plants where pre-germinated seedlings were planted into the *T. basicola*-containing soil than in the control plants where seeds were planted directly (Figure 9). As the disease levels in the treated seedling were similar for both pre-colonised seedlings or those grown from seed, the suppressive effect of *A. brasilense* was proportionally higher in the plants grown from seed.

This experiment demonstrated that *A. brasilense* can protect plants in a soil system when applied at the time of seed plantation. The invitro system was using sterile soil which eliminated the effects of large number of other soil biota. Field trials were then conducted to confirm the potential of *A. brasilense* as biocontrol agent in *T. basicola*-infested cotton fields.

PEM *and* A. brasilense *suppress black root rot symptoms to a similar degree invitro*

Invitro analysis of disease severity in cotton seedlings, grown in the presence of T. basicola and treated with either A. brasilense or the PEM bacteria at seed planting, indicated that PEM confers a similar level of protection to cotton seedlings against black root rot as A. brasilense under the conditions used (Figure 10).

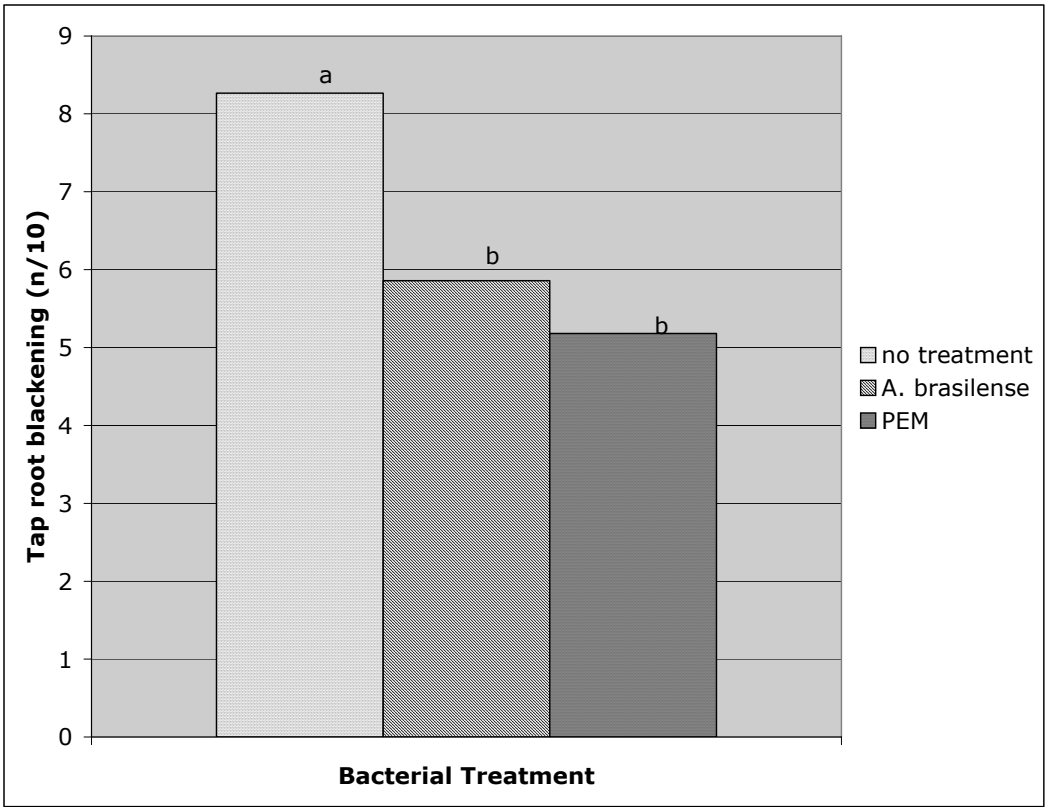
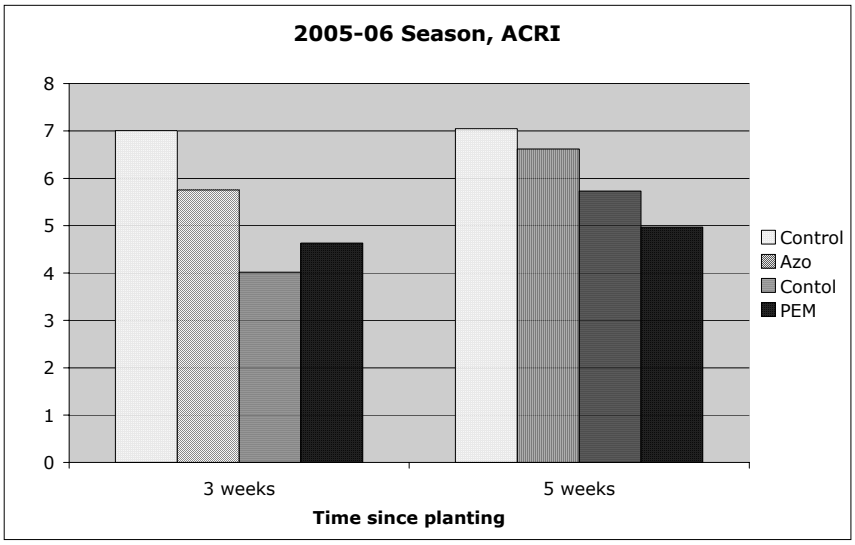


Figure 10: Tap root blackening in a pot trial. Seedlings were treated at planting with either *A. brasilense* or PEM. a,b indicate significant differences between treatments where p<0.01 (n=15,14,12)

There was a high degree of tap root blackening in the control plants that had received no treatment other than sterile H_2O after 13 days when grown in the presence of *T. basicola*, with almost the entire length of tap root showing the blackening characteristic of *T. basicola* infection (mean = 8.26). Statistical analysis of the results using a one-way ANOVA showed plants treated with either *A. brasilense* (mean = 5.86) or PEM (mean = 5.18) showed significantly less tap root blackening than untreated plants ($p<0.01$).

Disease suppression by PEM *and A. brasilense under field conditions*

Aggregated results from disease surveys conducted three and five weeks post-planting show that in all cases, the bacterial treatments had no significant effect. In no case was the disease level significantly increased by the bacterial treatments (Figure 11). Disease levels for individual plants were highly variable resulting in large standard deviations for the data.


2005-06 Season, ACRI
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6
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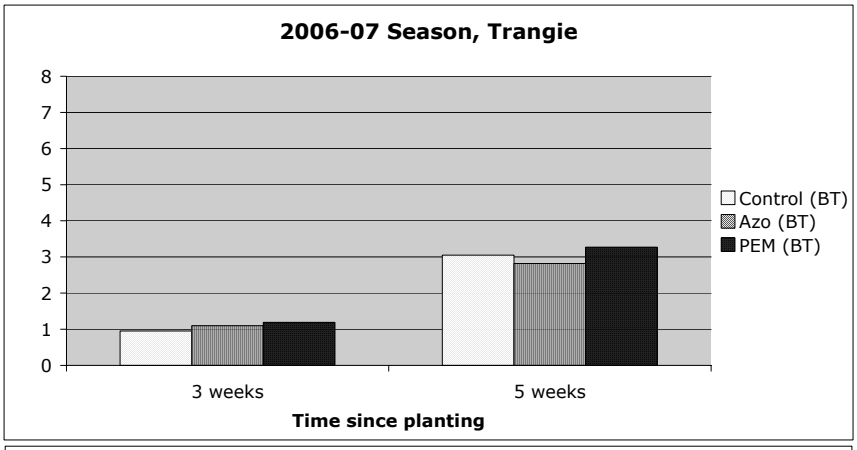
3 weeks
5 weeks
Time since planting
Control
Azo
Contol
PEM


2006-07 Season, Trangie
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Time since planting
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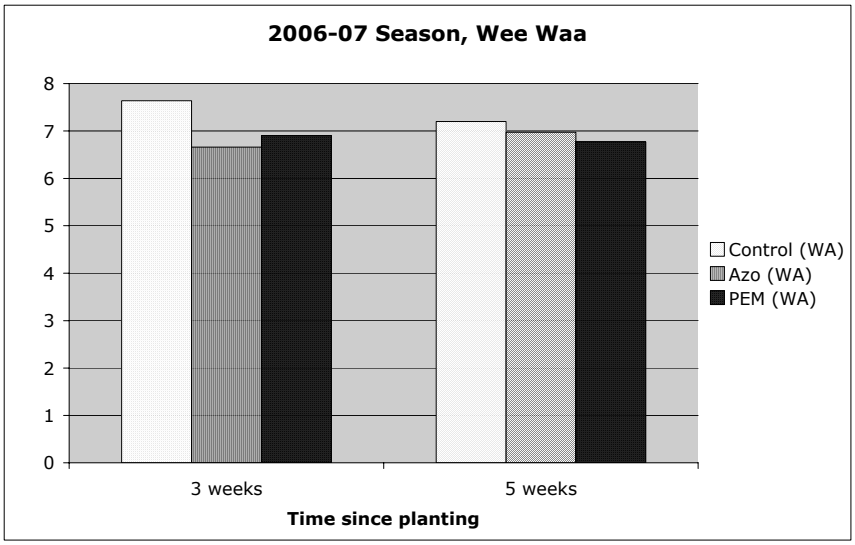
Azo (BT)
PEM (BT)


2006-07 Season, Wee Waa
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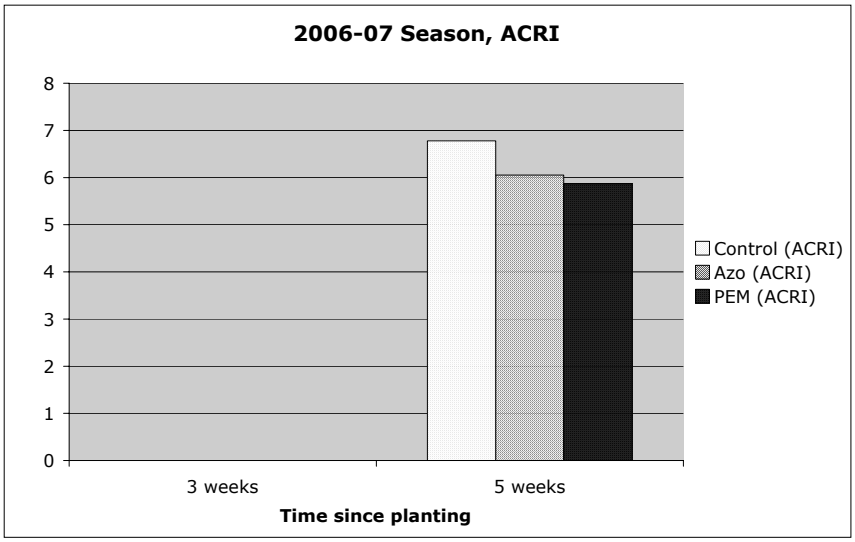

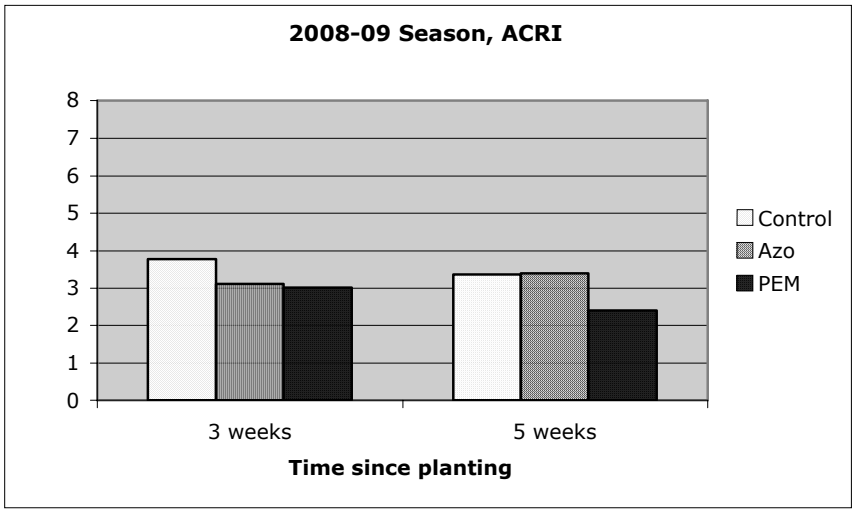

Figure 11: Field trials using *A. brasilense* (Azo) or PEM as planting inoculants. Tap root blackening due to *T. basicola* was assessed at three and five weeks post planting ACRI = Australian Cotton Research Institute, Narrabri NSW; WA = Wee Waa, NSW; BT = Trangie, NSW. (No significant differences were observed).

3.5. Discussion

A. brasilense has been used for many years as a plant growth promoting bacteria in crops including maize, wheat, sorghum and millet (Bashan 1998; Fulchieri & Frioni 1994; Okon & Labandera-Gonzalez 1994) and more recently as a biocontrol agent in tomato growing systems and with *Arabadopsis thaliana* (Bashan & De-Bashan 2002; Romero et al. 2003). This species is naturally found in Australian soils (Sung & New 1998) and can increase plant growth through fixation of nitrogen (Roper & Ladha 1995). Recently, it has also been suggested that *A. brasilense* can protect plants from disease by inducing a systemic host response (Ramos Solano et al. 2008).

A. brasilense has previously been shown to have an effect in reducing black root rot symptoms caused by *T. basicola*, when allowed to pre-colonize cotton seedling root tissue prior to exposing them to disease pressure (Pereg, Unpublished). The pre-germinated seedlings have had more time to establish healthy roots before being exposed to disease and so their root system would be expected to respond more quickly to the disease challenge. Application of *A. brasilense* to soil has also been shown to be effective in reducing leaf spot (caused by *Pseudomonas syringae* pv. tomato DC3000) in *A. thaliana* (Ramos Solano et al. 2008). Pot trials confirmed that *A. brasilense* also inhibits development of *T. basicola* induced black root rot in cotton seedlings when applied to the seed at planting. Similar levels of disease suppression as that shown in cotton seedlings pre-inoculated with *A. brasilense* gave more evidence to suggest that this species would be effective at reducing black root rot symptoms under field conditions.

Disease suppression in cotton seedlings by PEM makes them a potential treatment for reducing black root rot. The PEM bacteria had previously been shown to be effective in reducing infection of wheat seedlings by *R. solani* AG-8, a soil-borne fungal pathogen, under laboratory conditions using field soil (Barnett et al. 2006). They have also been shown to inhibit the directional growth of *T. basicola* hyphae towards cotton seedlings (Moulynox, Chapter 2). Individually, *P. agglomerans* has been shown to inhibit post-harvest *Penicillium* mold in oranges (Teixidó et al. 2001) and reduce the incidence of fireblight in apples (Stockwell et al. 2002). *Microbacterium* spp. have been implicated in suppression of *Pythium* damping off (Postma et al. 2005) and bacterial blight (Fukui et al. 1999). Barnett et al (2006)

appears to be the first published data using *E. acetylicum* as a biocontrol species in plants. This published data made the PEM species good candidates for further testing against *T. basicola*. The results in this study show that under the controlled conditions used in this pot trial, the PEM bacteria provided a similar level of suppression of *T. basicola* as *A. brasilense*, which had already been selected as a potential biocontrol agent. Although in laboratory plate trials any combination of two of the PEM bacteria were effective, those trials were under controlled sterile conditions. It was expected that under the more complex conditions in field soil the bacteria might not survive as well and so all three bacteria were applied to maximise the chance to observe an effect on disease levels.

Although a range of cotton seed cultivars were used in the field trials (Table 4), previous reports have suggested there is no variation in resistance to black root rot between the cotton cultivars available (Wang & Davis 1997) and no other published evidence that any cotton cultivar is more resistant was available so all cultivars were treated equally.

The data at many sites showed a trend towards lower mean disease levels, however, no significant difference between treated and untreated cotton were observed when applying the bacterial treatments in the field. In no case did any treatment lead to an increased level of disease. The lack of significant data is partially explained by the field trial design. Although 60 plants were assessed at each collection point, the block design means that data has to be analysed as 6 replicates of 10 plants, rather than 60 replicates of 1 plant. This means that variability between blocks increases the error term during data analysis and so potentially small significant differences are obscured by the natural variability across a field. To improve the data, a larger data set would be needed to increase the number of replicates and increase the chance of detecting any significant variations.

A. brasilense has been used with some success in other cases as a growth promoting bacteria (Fulchieri & Frioni 1994; Okon & Labandera-Gonzalez 1994). It was not determined in this study if application of *A. brasilense* at planting leads to increased growth later in the season, however, it is possible that the plants may show increased yield due to the effect of *A. brasilense* as a plant growth promoting

species. PEM and *A. brasilense* should be tested as a combined application which may prove more effective.

The relatively small areas planted for the field trials, and the destructive nature of the disease level testing, did not allow measurements of yield. Further work using larger scale trials is now required to test for a yield difference at harvest. If a yield difference is observed, then there is potential for development of a product for large-scale application.

3.6. Acknowledgements

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3.7. References

Allen SJ (1990) *Thielaviopsis basicola*, a new record on cotton in Australia. *Australasian Plant Pathology* **19**, 24-25.

Allen SJ, Nehl DB, Moore N Integrated Disease Management. In. pp. 17-18. (Australian Cotton Cooperative Research Centre)

Allen SJ, Nehl DB, Moore N (2008) 'Integrated Disease Management.' Australian Cotton Cooperative Research Centre.

Barnett SJ, Roget DK, Ryder MH (2006) Suppression of Rhizoctonia solani AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and *Microbacteria*. *Australian Journal of Soil Research* **44**, 331-342.

Bashan Y (1998) Inoculants of Plant Growth-promoting Bacteria for use in Agriculture. *Biotechnology Advances* **16**, 729-770.

Bashan Y, de-Bashan LE (2002) Protection of Tomato Seedlings against Infection by *Pseudomonas syringae* pv. Tomato by Using the Plant Growth-Promoting Bacterium *Azospirillum brasilense*. *Applied and Environmental Microbiology* **68**, 2637-2643.

Fukui R, Fukui H, Alvarez AM (1999) Suppression of Bacterial Blight by a Bacterial Community Isolated from the Guttation Fluids of Anthuriums. *Applied and Environmental Microbiology* **65**, 1020-1028.

Fulchieri M, Frioni L (1994) *Azospirillum* inoculation on maize (*Zea mays*): effect on yield in a field experiment in central Argentina. *Soil Biology and Biochemistry* **26**, 921-923.

Jetiyanon K, Kloepper JW (2002) Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biological Control* **24**, 285-291.

Leeman M, Pelt JAv, Ouden FMd, Heinsbroek M, Bakker PAHM, Schoippers B (1995) Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of Pseudomonas fluorescens. *Phytopathology* **85**, 1021-1027.

Lindow SE (1988) Lack of correlation of In vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. *Ecology and Epidemiology* **78**, 444-450.

Liu L, Kloepper JW, Tuzun S (1995) Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria. *Phytopathology* **85**, 843-847.

Nehl DB, Allen SJ, Mondal AH, Lonergan PA (2004) Black root rot: a pandemic in Australian cotton. *Australasian Plant Pathology* **33**, 87-95.

Okon Y, Labandera-Gonzalez CA (1994) Agronomic applications of *Azospirillum*: An evaluation of 20 years of worldwide field inoculation. *Soil Biology and Biochemistry* **26**, 1591-1601.

Postma J, Geraats BPJ, Pastoor R, van Elsas JD (2005) Characterization of the Microbial Community Involved in the Suppression of *Pythium aphanidermatum* in Cucumber Grown on Rockwool. *Phytopathology* **95**, 808.

Ramos Solano B, Barriuso Maicas J, Pereyra de la Iglesia MT, Domenech J, Gutierrez Manero FJ (2008) Systemic Disease Protection Elicited by Plant Growth Promoting Rhizobacteria Strains: Relationship Between Metabolic Responses, Systemic Disease Protection, and Biotic Elicitors. *Phytopathology* **98**, 451-457.

Reddy MS, Hynes RK, Lazarovits G (1994) Relationship between in vitro growth inhibition of pathogens and suppression of preemergence damping-off and postemergence root rot of white bean seedlings in the greenhouse by bacteria *Canadian Journal of Microbiology* **40**, 113-119.

Romero AM, Correa OS, Moccia S, Rivas JG (2003) Effect of Azospirillum-mediated plant growth promotion on the development of bacterial diseases on fresh-market and cherry tomato *Journal of Applied Microbiology* **95**, 832-838.

Roper MM, Ladha JK (1995) Biological N2 fixation by heterotrophic and phototrophic bacteria in association with straw. *Plant and Soil* **174**, 211-224.

Stockwell VO, Johnson KB, Sugar D, Loper JE (2002) Antibiosis Contributes to Biological Control of Fire Blight by *Pantoea agglomerans* Strain Eh252 in Orchards. *Phytopathology* **92**, 1202-1209.

Sung OH, New PB (1998) Isolation of Azospirillum spp. from natural soils by immunomagnetic separation. *Soil Biology and Biochemistry* **30**, 975-981.

Teixidó N, Usall J, Palou L, Asensio A, Nunes C, Viñas I (2001) Improving Control of Green and Blue Molds of Oranges by Combining *Pantoea Agglomerans* (CPA-2) and Sodium Bicarbonate. *European Journal of Plant Pathology* **107**, 685-694.

Wang H, Davis RM (1997) Susceptibility of Selected Cotton Cultivars to Seedling Disease Pathogens and Benefits of Chemical Seed Treatments *Plant Disease* **81**, 1085-1088.

Ward WT, McTainsh G, McGarry D, Smith KJ (1999) The soils of the Agricultural Research Station at 'Myall Vale', near Narrabri, NSW with data analysis by fuzzy k-means. In.

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4 A pilot study to assess three soil amendments for potential use in the field to suppress *Thielaviopsis basicola*

4.1. Abstract

Various soil amendments have been used to suppress fungal pathogens. Three readily available soil amendments, yeast extract, zein and soy protein were tested in a pot trial to determine their ability to reduce survival of *Thielaviopsis basicola* spores. Disease symptoms in cotton seedlings planted in soil infested with *T. basicola* spores and amended with zein or soy protein three weeks prior to planting were not significantly reduced. Yeast extract reduced disease symptoms to zero when soil was sterilised prior to addition of *T. basicola* endoconidia and amendment, but not when live soil was used. None of these amendments are recommended for application in field trials.

4.2. Introduction

In a review of published data, many different soil amendments, including sewage sludge, composted bark and sugarcane waste have been suggested to inhibit soil-borne diseases including Fusarium wilt, Rhizoctonia root rot and Phytophthora crown rot through stimulation of antagonistic microbial populations (Whipps 1997). Another study showed liquid swine manure, and meat and bone meal could reduce potato scab caused by *Verticillium dahliae* through production of ammonia, nitrate and nitrite during breakdown or changing the soil pH (Lazarovits et al. 2001).

Plant based amendments, where a winter cover crop is ploughed into soil before planting cotton, have been assessed for their effectiveness against *T. basicola*. Candole and Rothrock (1997) confirmed that incorporation of hairy vetch into soil prior to planting reduced the *T. basicola* chlamydospore viability in the field. Indian mustard has also been proposed as a ‘green manure’ crop (Bates & Rothrock 2005) to inhibit black root rot. In Australia, large areas of cotton growing regions

have been either officially in drought or marginal over the past six years (*Current Drought Situation* 2008), and so fields are frequently left fallow in winter to minimise water use. Therefore, although these plant based amendments may be effective, they are not always practical.

At less than 100 cfu/g soil there is a significant decrease in black root rot incidence in cotton crops (Nehl et al. 2004), and so substances that could decrease the viability of *T. basicola* spores in the soil were sought. Only one paper could be found that directly addressed using non-plant soil amendments to inhibit *T. basicola*. Papavizas and Adams (1969) suggested that the addition of fatty acids, lecithin, soybean protein, zein, yeast extract and chestnut tannin could all reduce the survival of spores of *T. basicola* in soil. Although some of these would not be practical for large scale field application, some of these substances are readily available and potentially cheap enough to develop into a solution for cotton growers. Soy flour is primarily composed of soy protein, zein is used in the confectionary industry as a glazing agent and yeast extract is used industrially in the production of products such as Vegemite™.

Although Papavizas and Adams (1969) were primarily interested in spore germination, they also tested viability of propagules after application of amendments. The three amendments chosen for this study all reduced the viability of propagules to zero when tested on culture plates. By extrapolation, if all the propagules are non-viable, they should not be able to infect cotton subsequently planted in the same soil.

The aim of this work was to test soy protein, zein and yeast extract for their effectiveness in suppressing black root rot symptoms in cotton seedlings through their ability to reduce viable spore numbers in soil when applied prior to planting.

4.3. Materials and Methods

T. basicola (BRIP40192) (Jan Dean, Q DPI&F) cultures were grown on half strength Potato Dexstrose Agar (½ PDA medium; 18.5g/L Bacto PDA powder and15g/L Difco agar) at 25°C for 4-6 days and kept refrigerated at 4°C until use.

Soil, classified as an alkaline grey-brown clacking clay, was collected from an active cotton growing field at ACRI (Narrabri, NSW) and sterilised by autoclaving three times with 24hr intervals. A sterile mixture of sand and soil (50:50w/w) was inoculated with *T. basicola* endoconidial spore suspensions, which were prepared according to the method described in section 2.2. The endoconidial suspension was added to the sterile sand:soil mix to give a final spore concentration of $1x10^3$ spores/g soil. Inoculated soil was placed into 200ml pots to be used in disease suppression experiments. Soil was then supplemented with either Zein, Soy protein or Yeast extract at a rate of 0.5%w/w or left without amendment (two cups per treatment), and then moistened to field capacity. The cups were covered with clear plastic film to minimise drying and incubated for three weeks at 23/18°C on a 12/12hr cycle. After three weeks, three cotton seeds, surface sterilised using 70% ethanol and 2% bleach (Moulynox, chapter 2) were planted in each cup and allowed to germinate. Pots were watered by soaking to maintain a high level of soil moisture. After incubation for a further three weeks, the plants were removed from the cups and their tap roots analysed for the presence of lesions caused by *T. basicola*. Pots containing non-germinated seeds were excluded from analysis.

A second pot trial, using non-sterile field soil was prepared. One set (three pots per treatment) of 200ml pots were prepared as before ($1x10^3$ endoconidia/g soil, 0.5% w/w amendments). A suspension of whole *T. basicola* culture was prepared by collecting the entire mycelial mat from a *T. basicola* culture plate, weighing, then suspending in several millilitres of sterile Milli-Q water and vortexing vigorously. A second set of pots was treated using this suspension (three pots per treatment) to give a final quantity of 10μg of mycelial mat per gram of soil. Pots were incubated then planted as previously described before assessing seedling for disease symptoms. Pots containing non-germinated seeds were excluded from analysis.

4.4. Results

Pre-incubation of soil with addition of yeast extract at 0.5% led to the T. basicola endoconidia being unable to infect germinating cotton seeds but only in the first

experiment (Table 5). In the second experiment, no treatment was effective in reducing disease levels (Table 6). Addition of zein or soy protein had no effect, with disease levels being not significantly different from the control plants (Table 6).

Overall mean disease levels were higher in the pots inoculated with mycelial mat as compared to pots containing endoconidia only (Table 6), however, due to the small number of plants, no statistical analysis was completed. Additionally, the mycelial mat could not be accurately enumerated for inoculum level.

Table 5: Disease levels in cotton seedling grown into sterile soil pre-treated and incubated with three different soil amendments at 0.5%w/w (n=5,3,5,6).

Treatment	**Mean disease level**
Control	3.2
Soy protein	2.7
Zein	2.0
Yeast extract	0.0

Table 6: Mean disease levels in cotton seedling planted into non-sterile soil pre-treated and incubated with three different soil amendments. Two different methods of inoculating soil with *T. basicola* were applied, using either a suspension of endoconidia only or a suspension of an entire colony (Mycelial mat) (Endoconidia only n=8,6,5,7; Mycelial mat n=9,8,8,7).

Treatment	Endoconidia only	Mycelial mat
Control	3.4	6.9
Soy Protein	1.0	6.5
Zein	6.5	5.4
Yeast Extract	4.0	6.6

4.5. Discussion

Papavizas and Adams (1969) observed viability of endoconidia in soil decreasing to three, zero and two percent (zein, yeast extract and soy protein) after seven days incubation for the three amendments under investigation. Their results with chlamydospores were similar, with zero percent viable propagules after seven days. If all (or nearly all) of the propagules are non-viable, they should not be able to infect cotton subsequently planted in the same soil. In the trial presented here, yeast extract was effective in reducing disease levels in one experiment in sterile soil, but the result was not replicated in non-sterile soil. Additionally, no reduction in disease symptoms was observed in the cotton seedlings challenged with both endoconidia and chlamydospores. Variability in performance of organic soil amendments have previously been reported to vary depending on soil type or even moisture conditions (Lazarovits 2001; Lazarovits et al. 2001). Regardless, performance in soil from an Australian cotton growing field is a requirement of potential amendments. These three amendments did not perform in the soil used in this experiment which was obtained from a cotton growing area that has *T. basicola* present.

It is difficult to compare dose rates for either the endoconidia or chlamydospores, as exact rates were not specified in the 1969 study though that study used “large numbers” and their control soil contained over $1x10^5$ propagules after one month, which is much greater than used in the endoconidial treatment in this study. As their

observations were of less than three percent viability after seven days, it would be expected that after three weeks incubation, our initial dose of *T. basicola* spores should be completely (or almost completely) inactivated, so observed disease levels whould also be expected to be close to zero.

The purpose of this study was to be a pilot study to determine whether these amendments could be applied under field conditions to reduce black root rot in cotton crops. All three of these amendments were eliminated as candidate soil treatments to reduce black root rot in the field, as although zein, yeast extract and soy protein have previously been shown to reduce *T. basicola* spore germination in soil (Papavizas & Adams 1969), these amendments did not protect cotton seedlings in live soil under controlled conditions. Yeast extract may have potential as an organic amendment as in one experiment in sterile soil, yeast extract reduced disease symptoms to zero, but this was not observed in the second experiment using non-sterile soil, and no inhibition of *T. basicola* was observed when using endoconidia and chlamydospores together as the disease challenge. As such, it could not be recommended to proceed to field trials with any of these amendments.

4.6. Acknowledgements

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4.7. References

Bates, G. & Rothrock, C.S. 2005, 'Potential use of high glucosinolate Indian mustard and canola for suppression of soilborne pathogens ', *Phytopathology,* vol. 95, no. 6, suppl., p. S7.

Candole, B.L. & Rothrock, C.S. 1997, 'Characterisation oif the Suppressiveness of Hairy Vetch-Ammended soils to *Thielaviopsis basicola*', *Phytopathology,* vol. 87, no. 2, pp. 197-202.

Current Drought Situation, 2008. Retrieved 16/1 2008 from http://www.dpi.nsw.gov.au/agriculture/emergency/drought/situation/drought-maps/drought-maps

Hood, M.E. & Shew, H.D. 1997, 'Reassesment of the role of saprophytic activity in the ecology of *Thielaviopsis basicola*', *Phytopathology,* vol. 87, no. 12, pp. 1214-1219.

Lazarovits, G. 2001, 'Management of soil-borne plant pathogens with organic soil amendments: a disease control strategy salvaged from the past ', *Canadian Journal of Plant Pathology,* vol. 23, pp. 1-7.

Lazarovits, G., Tenuta, M. & Conn, K.L. 2001, 'Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops', *Australasian Plant Pathology,* vol. 30, no. 2, pp. 111-117.

Nehl, D.B., Allen, S.J., Mondal, A.H. & Lonergan, P.A. 2004, 'Black root rot: a pandemic in Australian cotton', *Australasian Plant Pathology,* vol. 33, pp. 87-95.

Papavizas, G.C. & Adams, P.B. 1969, 'Survival of root-infecting fungi in soil XII. Germination and survival of endoconidia and chlamydospores of *Thielaviopsis basicola* in fallow soil and in soil adjacent to germinating bean seed', *Phytopathology,* vol. 59, pp. 371-378.

Whipps, J.M. 1997, 'Biological Control of Soil-borne Plant Pathogens', in *Advances in Botanical Research*, Academic Press, Birmingham, 26, pp. 1-134.

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5 Rs-AFP2, a peptide from radish seed, as a potential inhibitor of black root rot

5.1. Abstract

Rs-AFP2 is a 5kDa defensin protein naturally found in radish seeds and shown to suppress a range of plant pathogenic fungi. Complete inhibition of *T. basicola* by heat treated protein extract from radish seeds was observed in this study, implicating that the heat stable Rs-AFP2 protein has a role in this suppression. To confirm the role of Rs-AFP2, the gene encoding Rs-AFP2 was then cloned into a commercial strain of the yeast *Kluyveromyces lactis*. Growth inhibitions assays confirmed that Rs-AFP2 inhibits *T. basicola* invitro, however *K. lactis* was determined to be an unsuitable host strain for production of this protein. Rs-AFP2 acts through interaction with glucosylceramide which is present in the cell membrane of *K. lactis* but not in *Saccharomyces cerivisae*. Further work on this protein could be conducted using either *S. cerivisae* or a glucosylceramide-negative *K. lactis* mutant.

5.2. Introduction

Black root rot is a fungal disease affecting cotton seedlings caused by the soil-borne pathogen *Thielaviopsis basicola*. A number of plant derived peptides are known to inhibit fungal pathogens. These peptides can be genetically transferred into other plants or used as anti-fungal agents invitro. Of particular interest in this study are defensins, that either induce changes in fugal hyphal morphology or reduce fungal hyphal growth (Broekart et al. 1995). Two examples of defensin proteins include An1, isolated from *Atriplex nummularia* (saltbush), which inhibits *Rhizoctonia solani*, *Verticillium dahliae* and *T. basicola* (Last & Llewellyn 1997) and a 5.5kDa peptide isolated from alfalfa, alfAFP that has been successfully expressed in potato, and has been found to inhibit *V. dahliae, Alternaria solani* and *Fusarium culmonorum* (Gao et al. 2000).

Another plant defensin, Rs-AFP2 has been shown to reduce infection of transgenic tobacco by *Alternaria longipes* (Terras et al. 1995) and inhibit growth of a range of fungi invitro (Terras et al. 1992). Radish seed extract, which contains Rs-AFP2, along with several other anti-fungal peptides, has been shown to inhibit *T. basicola* (Last &

Llewellyn 1997) and purified Rs-AFP2 has been shown to inhibit a range of fungal pathogens (Terras et al. 1992) but inhibition of *T. basicola* by Rs-AFP2 has not been definitively demonstrated.

Rs-AFP2 reduces fungal growth by permeabilising the cell membrane (Thevissen et al. 1999) through a specific interaction with phospholipids contained in the cell wall of many fungi (Thevissen et al. 2004). Additionally, Rs-AFP2 does not interact with phospholipids derived from plants or human cells (Thevissen et al. 2004), making it safe to work with and unlikely to have phytotoxic effects. In fact, expression of the Rs-AFP2 gene in tomato and canola plants has been shown to reduce the effect of a number of pathogens (Parashina et al. 2000).

The ability of the Rs-AFP2 peptide to reduce growth of *T. basicola* invitro is investigated here using a commercial yeast based protein expression system as a method of producing large quantities of relatively pure protein without complex purification.

5.3. Materials and methods

Preparation and analysis of crude protein extract from radish seeds

Twenty five grams of *Raphanus sativus* (radish) seeds were ground in liquid nitrogen and a partial selective protein extraction was performed according to the method described by (Terras et al. 1992), excluding the last steps of dialysis and column separation. Rs-AFP2 is heat stable and so this method removes all heat labile proteins from the extract. A portion of the crude protein extract was further separated on a Centricon 10 spin column (Amicon) to exclude proteins smaller than 10kDa. Samples of the partially purified extract were mixed with 2x LUG loading buffer (2x LUG; 0.000125M Tris/Hcl, pH 6.8, 2% sodium dodecyl sulphate, 5% glygerol, 10% b-mercaptoethanol, 0.004% bromophenol blue) and analysed using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel (Ausubel et al. 1988).

Fungal growth inhibition testing

T. basicola (BRIP40192) cultures were grown on solid ½ PDA plates (containing 18.5g/L Bacto PDA medium, 15g/L Difco agar) at 25°C for 4-6 days. Culture plates were then kept at 4°C until use.

Potato dextrose broth (PDB) was prepared by boiling 220g washed and cut potatoes in 1L of water. The boiled solution was then filtered through Miracloth (Calbiochem) and 20g of dextrose was added. Volume was adjusted to 1L and then the solution was sterilised by autoclaving.

Endoconidial spore suspensions were prepared by scraping the entire mycelial mat from a ½ PDA *T. basicola* culture plate into approximately 5 ml of sterile PDB. This suspension was then vortexed vigorously for 1 min before filtering through two layers of miracloth (pre-soaked with 70% ethanol and dried under UV light) to exclude hyphal fragments and chlamydospore chains. Spore concentration was determined by direct count using a heamocytometer. The suspension was then diluted with PDB as required to give a final concentration of $1x10^4$ endoconidia/ml.

Aliquots of 95µl of endoconidial spore suspension were added to wells in 96 well plates. Five microlitres of treatments or sterile distilled water (for control) was then added and mixed thoroughly. Treatments were radish seed protein extract, *Kluyveromyces lactis* culture supernatants or whole cell protein extracts (see below). Plates were sealed with Parafilm to prevent evaporation and incubated in the dark at 25°C. Plates were periodically assessed for fungal growth using an automated plate reader (Titertek multiscan PLUS) at OD492nm.

Cloning of the RS-AFP gene into an expression vector

The Rs-AFP2 gene was assembled from eight oligonucleotides using an assembly PCR process. Four oligonucleotides, designated F1, F2, F3 and F4, ranging in size from 54 to 74 bases were synthesised (GeneWorks) covering the coding sequence of the Rs-AFP2 gene with the addition of several nucleotides at the 5' end of the first oligonucleotide (F1) to add an *Xho*I site and a Kex processing site. Extra nucleotides were added at the 3' end of the fourth oligonucleotide (F4) to add a *Bgl*II site. The

two restriction sites were chosen to allow cloning into the pKLAC1 vector (NEBS). Four complementary oligonucleotides were then synthesised (GeneWorks) covering the reverse sequence and designated R1, R2, R3 and R4. The eight oligonucleotides were designed such that each complimentary oligonucleotide pair (eg F1 and R1) would have an overhanging end complementary to the next oligonucleotide pair (eg F2 and R2) (see Appendix 1 for sequence data).

A two stage PCR reaction was performed. The first reaction (PCR A) was designed to assemble the fragments into a continuous template that was then used in the second reaction (PCR B) to generate a large quantity of double stranded Rs-AFP2. For PCR A, a 20μl reaction mix was prepared using 2.5μl of oligonucleotide mix (1pmol/μl of each oligonucleotide F1-F4, R1-R4), 2μl 10x Taq buffer (NEBS), 1μl dNTP mix (Promega)(each NTP at 10mM) and 2.5U Taq polymerase (NEBS). The reaction was performed in a standard thermocyler (MJ Research Inc., PTC-100). The reaction tube was heated to 95°C for 1 minute to ensure complete denaturation of all oligonucleotides followed by 12 cycles of 94°C, 30 seconds, 52°C, 30 seconds, 72°C 1 minute. A 50μl reaction was then prepared (PCR B) using 0.5μl of reaction PCR A as the template, 5μl 10x Taq buffer, 2μl dNTP mix (each NTP at 10mM), 5U Taq polymerase, 1μl forward primer (AFP-Primer 1; CGGTCTCGAGAAAAGAATG) (20pmol/μl) and 1μl of reverse primer (AFP-Primer 2; GCCGAGATCTTTAACTAGG) (20pmol/μl). A 1min denaturation step was then performed at 94°C followed by 30 cycles of 94°C, 30 seconds, 52°C, 30 seconds, 72°C 1 minute. A final step of 72°C for 5 minutes was added to increase the quantity of the full length product produced. This reaction was then run on an agarose gel and the resulting 255bp band excised and purified using the QIAquick PCR purification kit (Qiagen, #28104) according to the manufacturers instructions, then digested with *Xho*I (Pharmacia) and *Bgl*II (Pharmacia) and purified again.

Ligation of the digested PCR fragment and the pKLAC1 plasmid (NEBS) was then prepared based on cloning strategy III of a *K. lactis* protein expression kit, (NEBS) with some modifications. In order to eliminate the risk of incompletely digested pKLAC1 plasmid self ligating and producing false positive colonies on selective media that did not contain the RS-AFP gene, a 3 fragment ligation was performed. Two separate digests of pKLAC1 were prepared, one digested with *Xho*I and *Sac*I

(NEBS) to yield 2667bp and 6424bp fragments, the other digested with *Bgl*II and SacI to yield 2634bp and 6457bp fragments. These digests were run on an agarose gel and the 6424bp *Xho*I/*Sac*I fragment and the 2634bp *Bgl*II/*Sac*I fragments excised and purified using the QIAEXII Gel extraction kit (Qiagen, #20021). The ligation was then prepared according to the manufacturers protocol using the three purified fragments (pKLAC1 *Xho*I/*Sac*I, pKLAC1 *Bgl*II/*Sac*I, PCR B *Xho*I/*Bgl*II). The result was plasmid pKLAFP2, containing the Rs-AFP2 gene inserted into the multiple cloning site of pKLAC1. Plasmids containing the Rs-AFP2 gene inserted into the multiple cloning site of pKLAC1 were selected in *E. coli* (JMX8634) on Luria-Bertani (LB) plates containing ampicillin (100ug/ml). Five colonies that formed (containing the pKLAFP2 plasmid) were harvested and the plasmid recovered using the High Pure Plasmid Isolation kit (Roche). The region of the plasmid expected to contain the Rs-AFP2 gene was then sequenced (Supamac).

None of five sequenced plasmids showed 100% homology to the Rs-AFP2 coding region. pKLAFP2-2 was a close match, containing a 4bp deletion at position 33 and a single base substitution at position 244 and the errors occurred near the ends of the sequence. A PCR reaction using oligonucleotides F1 and R4 as primers was performed using the PCRB protocol and pKLAFP2-2 as the template DNA. pFU polymerase (Promega) was used in this reaction due to its inherent error checking ability. The PCR product was digested and ligated as previously described above to yield plasmid pKLAFPr. *E. coli* JMX8634 cells were transformed, several colonies selected, plasmids harvested and sequenced. Plasmids were designated pKLAFPr(n).

Genes cloned in plasmids pKLAFPr3 and pKLAFPr8 showed 100% homology to the Rs-AFP2 coding region. These two plasmids were linearised with *Sac*II (NEBS) and transformed into competent *K. lactis* cells using the *K. lactis* Protein Expression Kit (NEBS #E1000S) according to the manufacturers instructions. As a control, a third plasmid, pKLAC1-malE1 containing the *E. coli* maltose binding protein was also transformed into *K. lactis*. All the transformed cells were selected on Yeast Carbon Base (supplied with kit) plates containing acetamide as the only source of nitrogen. Only cells containing the integrated DNA can utilise acetamide as a nitrogen source. Several colonies were patched onto fresh YCB plates and stored at 4°C. Additional

samples were also prepared and stored in 50% glycerol at -70°C. As required, colonies selected for further analysis were then grown in YP Gal (10g/L yeast extract, 20g/L Bacto Peptone, 20g/L galactose).

PCR Confirmation of insertion of the Rs-AFP2 gene into the K. lactis genome

Selected transformed *K. lactis* colonies were inoculated into 5 ml of fresh YP Gal and incubated for 48hrs at 30°C. Cultures were centrifuged and the supernatant discarded. Cells were resuspended in 200µl of lysis buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCL, pH 8, 1mM EDTA, pH 8) and DNA harvested using the Bust'n'Grab protocol (Harju et al. 2004) with the following modifications: the snap freezing step was performed in a 100% ethanol bath at -70°C; centrifugation steps were performed at 14000g. Samples were air dried before being resuspended in 30µl of TE buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and stored at 4°C until use.

Primers corresponding to the start (AFPF-Start; ATGGCTAAGTTTGCTTCTATC) and stop codons (AFPR-Stop; TTAACAAGGGAAATAACAG) of the Rs-AFP2 gene were synthesised. A PCR reaction was performed using the PCR B protocol previously described, changing the annealing temperature to 45°C (primer Tm were 48°C and 42°C) and using the harvested genomic DNA of the transformed *K. strain* (expected to contain the Rs-AFP2 gene) or pKLAFPr3 (whose coding region had previously been sequenced) as template. The resulting PCR products were run on a 1.2% agar gel and compared.

Testing clones containing the Rs-AFP2 gene insert for anti-fungal activity

K. lactis colonies containing either the Rs-AFP2 insert (treatment) or the malE1 insert (control) were grown in 10 ml YPGal media at 30°C at 250RPM. After 5 days growth, 20ml cultures were centrifuged and the supernatant was harvested and stored at 4°C (less than 1 week) or -20°C (up to 1 month) until use. The resulting pellet was

resuspended in 1ml of 10mM sodium azide and whole cell protein extracted according to the method developed by Hoffman et al. (2002). The resulting protein extract was stored at 4°C (less than 1 week) or -20°C (up to 1 month) until use.

The supernatant and the whole cell protein extracts were tested for anti-fungal activity in 96 well plates containing a suspension of *T. basicola* endoconidial cells. Five microlitres of either PDB (control), supernatant or whole cell protein extract were added to each well and fungal growth measured spectrophotometrically on an automated plate reader (Titertek multiscan PLUS) at OD492nm.

5.4. Results

Inhibition of T. basicola by partially purified radish seed extracts

Analysis of the protein extract obtained from radish seeds on the SDS-PAGE gel yielded three bands (Figure 12). Two of the bands, 5kDa and 20kDa, correspond with bands identified by Terras et al. (1992) as belonging to Rs-AFP2. The 2.5kDa band was unidentified, however, this extract is expected to contain multiple proteins. The purification process removes the majority of unwanted proteins by ammonium sulfate precipitations. Further unwanted proteins are removed by heating the sample as Rs-AFP2 is not degraded by heat. Several other identified heat stable proteins still remain, including Rs-AFP1 and the 2S albumin proteins. A sample of the extract was spun on a Centricon 10 spin column, to remove any proteins smaller than 10kDa, and run on another gel (Figure 13). The same banding pattern was observed. As Rs-AFP2 is suspected to be a tetramer of 5kDa subunits it was thought that the 20kDa band may have been due to incomplete separation of subunits. After further processing to eliminate any proteins of less than 10kDa, the spun sample gave the same band pattern as before suggesting that the smaller bands may be subunits of the tetrameric protein that is intact in solution, but is separated when placed in the denaturing gel.

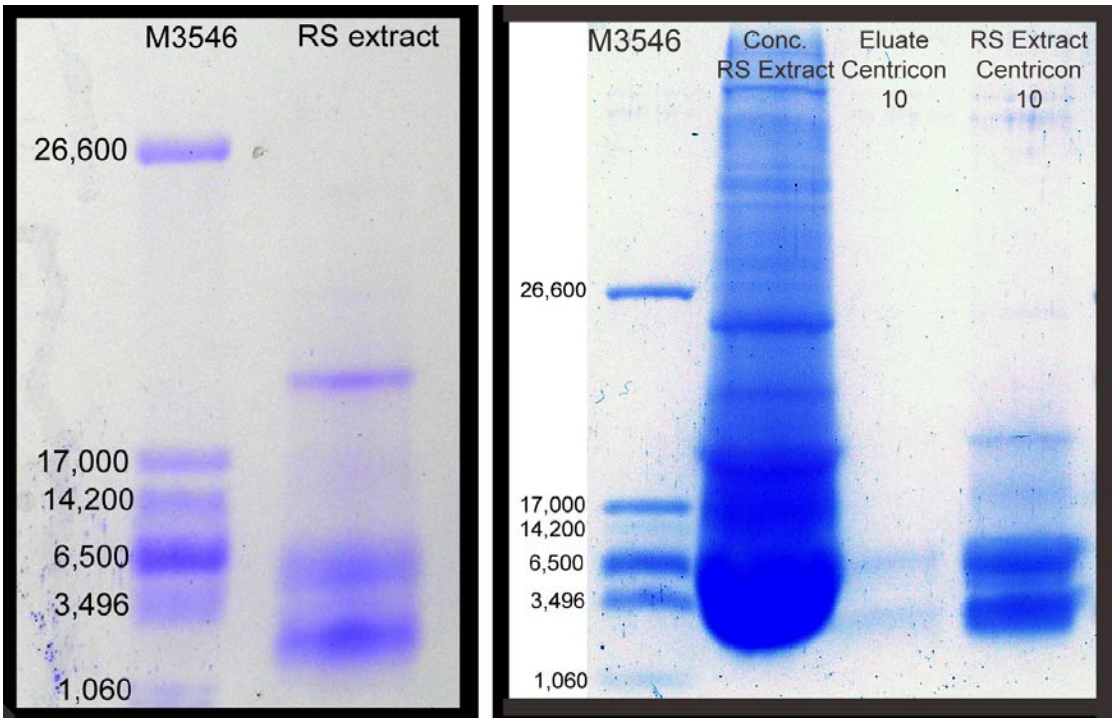

Figure 12: (Left image) Partially purified *Raphanus sativus* extract was run on an SDS-PAGE. Bands of approximately 2.5kDa (unidentified), 5kDa (Rs-AFP2) and 20kDa (Rs-AFP2 tetramer) are clearly visible (right lane) when compared to the molecular weight standard (left lane)

Figure 13: (Right image) Partially purified *R. sativus* extract after processing on a Centricon-10 column. Lane 1 molecular weight standard, Lane 2 concentrated sample, Lane 3 eluate, Lane 4 centrifuged sample showing the same banding pattern as the sample in Figure 1.

Partially purified radish seed extract containing Rs-AFP2 completely inhibited *T. basicola* endoconidial germination, with no germ tubes microscopically visibly after 48 hours incubation (Figure 14). In control wells containing no protein extract, most spores had germinated and extended germ tubes (Figure 14).

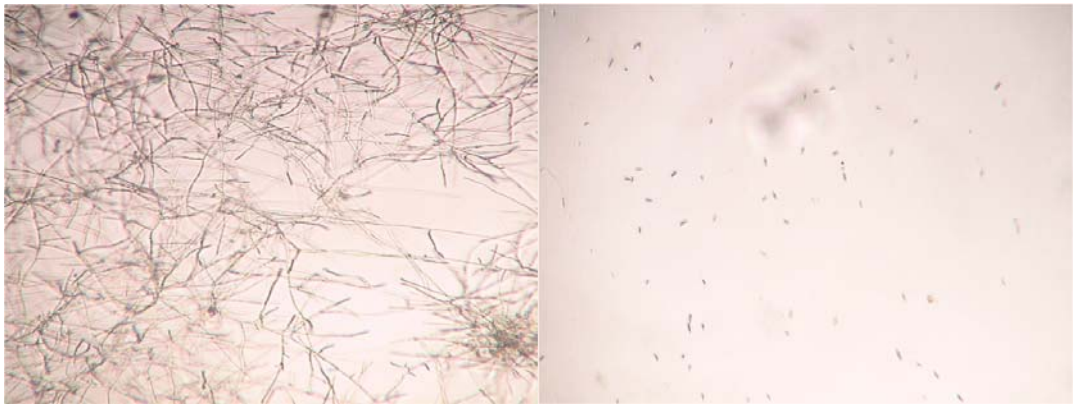

Figure 14: Endoconidial germination under high nutrient conditions (PDB) after 48 hours incubation in the presence (right) and absence (left) of a small quantity of protein extract from *R. sativus*.

Cloning of the Rs-AFP2 gene

In order to confirm that the source of the inhibition of *T. basicola* observed in the previous experiment was indeed the Rs-AFP2 protein, further purification of the protein was required. The Rs-AFP2 gene was assembled and inserted into the multiple cloning site of the pKLAC1 plasmid to yield pKLAFP2. The resultant plasmid Rs-AFP2-2 had the closest homology with the Rs-AFP2 sequence, containing a 4bp deletion at position 33 and a single base substitution at position 244 (Figure 16). These two errors were repaired using PCR and further plasmids were created using the corrected insert. Of five plasmids whose sequence was obtained, pKLAFPr3 and pKLAFPr8 showed 100% homology to the Rs-AFP2 gene sequence (Figure 17). pKLAFPr0, pKLAFPr4 and pKLAFPr6 contained errors outside the coding sequence in the cloning sites that prohibited using them further. Following cloning of these two plasmids into *K. lactis* and subsequent screening, six clones (designated 31, 32, 81, 82, 83, 84) were confirmed to have the AFP gene insert, with a 255bp band produced in PCR amplification using primers AFPF-Start and AFPR-Stop, corresponding to the same band produced when using the pKLAFPr3 plasmid as a template (Figure 15).

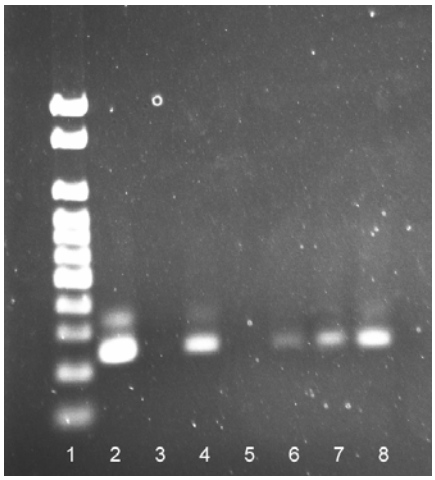

Figure 15: PCR to confirm the presence of the Rs-AFP2 gene. Lane 1, 100bp ladder; Lane 2, pKLAFPr3 (control); Lane 3, clone M1; Lane 4, clone 31; Lane 5, clone M2; Lane 6, clone 32; Lane 7, clone 81; Lane 8, clone 82. (Clones 83 and 84 not shown on this gel).

```
                                  1         10        20        30        40        50        60
                                  |         |         |         |         |         |         |
RS-AFP2 gene coding sequence      ------atggctaagtttgcttctatcattgtc  tcttcgttgctcttgtcgttttt
                      RS-AFP2-2   AAAAGAATGGCTAAGTTTGCTTCTATCATTGTC  TCTTCGTTGCTCTTGTCGTTTTT
                      RS-AFP2-4   AAAAGAATGGCTAAGTTTGCTTCTATCATTGTCCTTCTCTTCGTTGCTCTTGTCGTTTTT

RS-AFP2 gene coding sequence      gctgctttcgaagaaccaacaatggtggaagcacagaagttgtgtcagaggccaagtggg
                      RS-AFP2-2   GCTGCTTTCGAAGAACCAACAATGGTGGAAGCACAGAAGTTGTGTCAGAGGCCAAGTGGG
                      RS-AFP2-4   GCTGCTTTCGAAGAACCAACAATGGTGGAAGCACAGAAGTTGTGTCAGAGGCCAAGTGGG

RS-AFP2 gene coding sequence      acatggtcaggagtctgtggaaataataacgcatgcaagaatcagtgcattcgacttgag
                      RS-AFP2-2   ACATGGTCAGGAGTCTGTGGAAATAATAACGCATGCAAGAATCAGTGCATTCGACTTGAG
                      RS-AFP2-4   ACATGGTCA-GAGTCTGTGGAAATAATAACGCATGCAAGAATCAGTGCATTCGACTTGAG

RS-AFP2 gene coding sequence      aaagcacgacatgggtcttgcaactatgtcttcccagctcacaagtgtatctgttatttc
                      RS-AFP2-2   AAAGCACGACATGGGTCTTGCAACTATGTCTTCCCAGCTCACAAGTGTATCTGTTATTTC
                      RS-AFP2-4   AAAGCACGACATGGGTCTTGCAACTATGTCTT--CAGCTCACAAGTGTATCTGTTATTTC

RS-AFP2 gene coding sequence      cct gttaa------
                      RS-AFP2-2   CCT GTTAAAGATCT
                      RS-AFP2-4   CCT GTTAAAGATCT
```

Figure 16: Sequence alignment showing the Rs-AFP2 coding sequence compared to the sequence of two putative Rs-AFP2 inserts. The sequence errors in Rs-AFP2-2 were near the start and end of the sequence and this clone was selected for further use.

```
                                1         10        20        30        40        50        60
                                |         |         |         |         |         |         |
RS-AFP2 gene coding sequence    ------atggctaagtttgcttctatcattgtccttctcttcgttgctcttgtcgttttt
                    RS-AFPr0    A-AAGAATGGCTAAGTTTGCTTCTATCATTGTCCTTCTCTTCGTTGCTCTTGTCGTTTTT
                    RS-AFPr3    AAAAGAATGGCTAAGTTTGCTTCTATCATTGTCCTTCTCTTCGTTGCTCTTGTCGTTTTT
                    RS-AFPr4    AAAAGAATGGCTAAGTTTGCTTCTATCATTGTCCTTCTCTTCGTTGCTCTTGTCGTTTTT
                    RS-AFPr6    AAAAGAATGACTAAGTTTGCTTCTATCATTGTCCTTCTCTTCGTTGCTCTTGTCGTTTTT
                    RS-AFPr8    AAAAGAATGGCTAAGTTTGCTTCTATCATTGTCCTTCTCTTCGTTGCTCTTGTCGTTTTT

RS-AFP2 gene coding sequence    gctgctttcgaagaaccaacaatggtggaagcacagaagttgtgtcagaggccaagtggg
                    RS-AFPr0    GCTGCTTTCGAAGAACCAACAATGGTGGAAGCACAGAAGTTGTGTCAGAGGCCAAGTGGG
                    RS-AFPr3    GCTGCTTTCGAAGAACCAACAATGGTGGAAGCACAGAAGTTGTGTCAGAGGCCAAGTGGG
                    RS-AFPr4    GCTGCTTTCGAAGAACCAACAATGGTGGAAGCACAGAAGTTGTGTCAGAGGCCAAGTGGG
                    RS-AFPr6    GCTGCTTTCGAAGAACCAACAATGGTGGAAGCACAGAAGTTGTGTCAGAGGCCAAGTGGG
                    RS-AFPr8    GCTGCTTTCGAAGAACCAACAATGGTGGAAGCACAGAAGTTGTGTCAGAGGCCAAGTGGG

RS-AFP2 gene coding sequence    acatggtcaggagtctgtggaaataataacgcatgcaagaatcagtgcattcgacttgag
                    RS-AFPr0    ACATGGTCAGGAGTCTGTGGAAATAATAACGCATGCAAGAATCAGTGCATTCNACTTGAG
                    RS-AFPr3    ACATGGTCAGGAGTCTGTGGAAATAATAACGCATGCAAGAATCAGTGCATTCGACTTGAG
                    RS-AFPr4    ACATGGTCAGGAGTCTGTGGAAATAATAACGCATGCAAGAATCAGTGCATTCGACTTGAG
                    RS-AFPr6    ACATGGTCAGGAGTCTGTGGAAATAATAACGCATGCAAGAATCAGTGCATTCGACTTGAG
                    RS-AFPr8    ACATGGTCAGGAGTCTGTGGAAATAATAACGCATGCAAGAATCAGTGCATTCGACTTGAG

RS-AFP2 gene coding sequence    aaagcacgacatgggtcttgcaactatgtcttcccagctcacaagtgtatctgttatttc
                    RS-AFPr0    AAAGCACGACATGGGTCTTGCAACTATGTCTTCCCAGCTCACAAGTGTATCTGTTATTTC
                    RS-AFPr3    AAAGCACGACATGGGTCTTGCAACTATGTCTTCCCAGCTCACAAGTGTATCTGTTATTTC
                    RS-AFPr4    AAAGCACGACATGGGTCTTGCAACTATGTCTTCCCAGCTCACAAGTGTATCTGTTATTTC
                    RS-AFPr6    AAAGCACGACATGGGTCTTGCAACTATGTCTTCCCAGCTCACAAGTGTATCTGTTATTTC
                    RS-AFPr8    AAAGCACGACATGGGTCTTGCAACTATGTCTTCCCAGCTCACAAGTGTATCTGTTATTTC

RS-AFP2 gene coding sequence    ccttgttaa------
                    RS-AFPr0    CCTTGTTAAAGATCT
                    RS-AFPr3    CCTTGTTAAAGATCT
                    RS-AFPr4    CCTAGTTAAAGATCT
                    RS-AFPr6    CCTTGTTAAAGATCT
                    RS-AFPr8    CCTTGTTAAAGATCT
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Figure 17: Sequence alignment showing the Rs-AFP2 coding sequence compared to the sequence of five putative Rs-AFP2 inserts. RS-AFPr3 and RS-AFPr8 both show 100% homology to the published sequence for the Rs-AFP2 gene.

Rs-AFP2 inhibits T. basicola invitro

The *K. lactis* expression system (NEBS) had been designed so that any protein produced should be excreted into the culture medium. As radish seed extract (RSE) completely inhibited the growth of *T. basicola* in previous tests, it was used as a positive control to see how well the treatments containing Rs-AFP2 inhibits endoconidial germination. A 96 well growth inhibition assay was prepared using whole cell protein extract from the six clones containing the Rs-AFP2 gene (31, 32, 81, 82, 83, 84) as well as two control clones containing the malE1 gene (M1 and M2) and spent culture medium obtained after growing the same clones, as treatments. PDB was used as an additional control treatment. Data is presented in two graphs

showing growth either in the presence of spent culture medium (Figure 18) or whole cell protein extract (FFigure 19). Spent culture medium from colonies 31, 32, 81, 82, 83 and 84 inhibited growth of *T. basicola* when compared to the supernatant of the control colonies (M1, M2) (Figure 18). When compared to the PDB control, however, overall growth in the treatment wells was higher. The second graph shows the growth result when the treatment added was a whole cell protein extract. Again, growth in the wells containing extract from the control colonies was higher than the PDB control, however growth in the wells containing extract from the positive clones was virtually zero (FFigure 19).

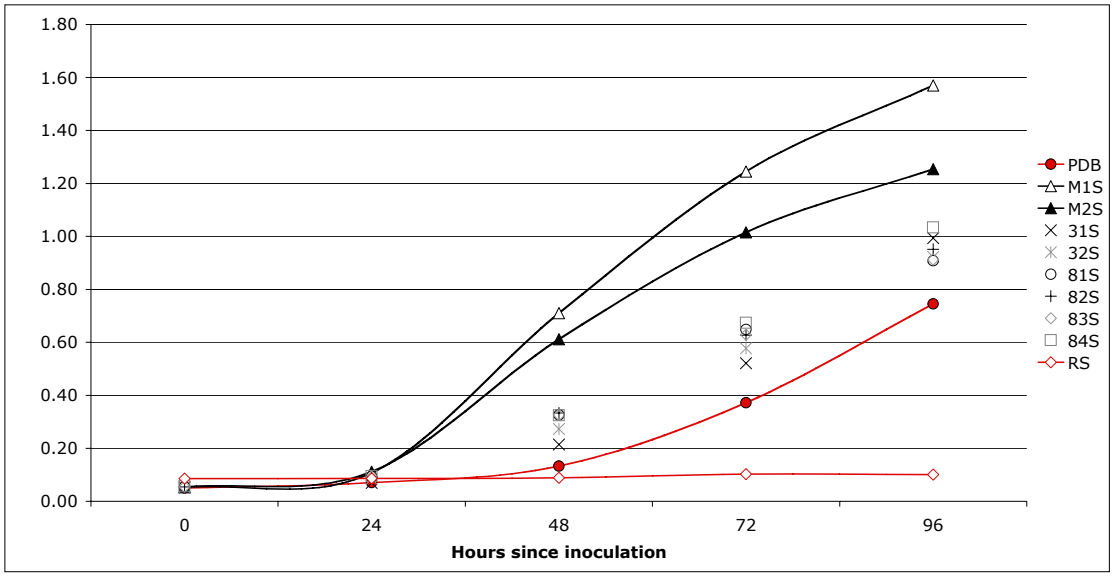

Figure 18: Spent culture medium from *K. lactis* cultures containing the Rs-AFP2 gene (31S, 32S, 81S, 82S, 83S and 84S) was added to wells containing *T. basicola* endoconidia and fungal growth compared to wells containing spent culture medium from colonies without the Rs-AFP2 gene (M1S and M2S) or PDB only. RSE was included as a negative control.

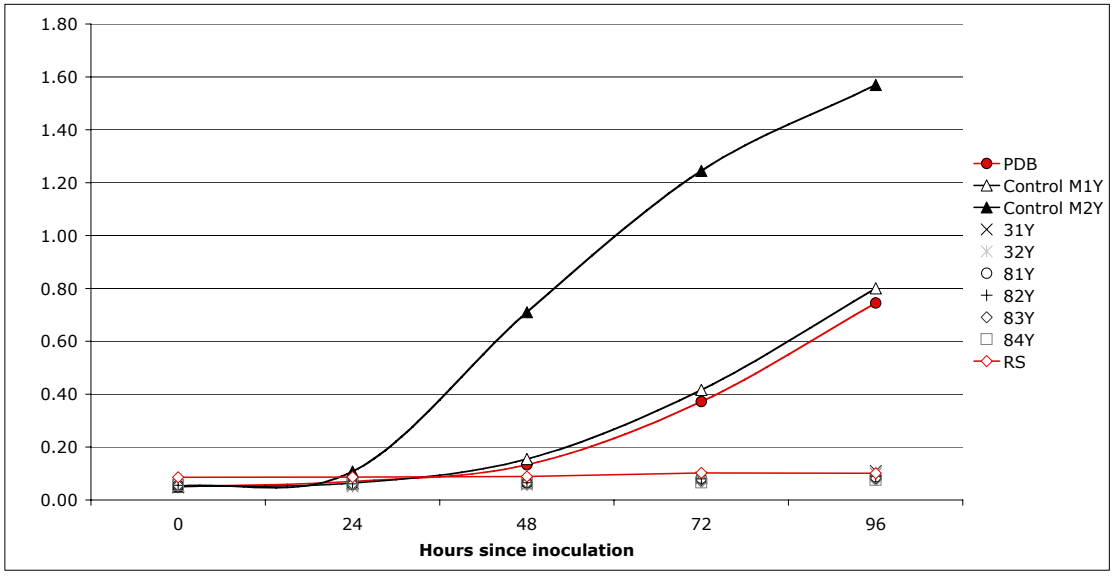

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Figure 19: Whole cell protein extract (WCE) from *K. lactis* cells containing the Rs-AFP2 gene (31Y, 32Y, 81Y, 82Y, 83Y and 84Y) or without the Rs-AFP2 gene (M1Y and M2Y) was added to wells containing *T. basicola* endoconidia and fungal growth compared. RSE was included as a negative control.

5.5. Discussion

Plants produce a range of proteins to defend against attack by pathogens. Not all of these are direct inhibitors of pathogenic micro-organisms. In a review of plant interactions with pathogens, Hammond-Kosack & Jones (1997) discuss a number of genes that confer resistance to specific pathogens by mechanisms other than direct inhibition. The various resistance or R genes they describe are suspected to only interact with pathogens as a method of detection and then activate other plant defence mechanisms such as production of callose material or salicylic acid. Other plant proteins, such as the defensins, interact more directly with pathogens to inhibit their growth or change their morphology (Broekart et al. 1995; Terras et al. 1992).

Rs-AFP2 is a protein that has been shown to inhibit a range of pathogenic fungi including *Verticillium dahliae* and two strains of *Fusarium oxysporum* (Terras et al. 1992). This protein was selected as a candidate for suppressing *T. basicola* both invitro and in Australian cotton growing fields for several reasons. Rs-AFP2 had previously been expressed in yeast (Terras et al. 1992), so it was expected that an expression system could be developed. The protein is heat stable, retaining anti-fungal activity after heating to 100°C (Terras et al. 1992). This is an important factor as any product developed using this protein could be exposed to temperatures in excess of 40°C during storage in Australian cotton growing regions in central western NSW (*Climate Averages* 2009). Rs-AFP2 was not expected to be toxic to either the plants or humans as it has been shown to interact only with fungal cell walls (Thevissen et al. 2004). Sequence data for the gene was available, making it possible to assemble the gene from oligonucleotides.

Rs-AFP2 had potentially been implicated as an inhibitor of *T. basicola* by the use of radish seed extract in growth inhibition assays (Last & Llewellyn 1997), but the extracts also contain other proteins known to inhibit fungal growth (Terras et al. 1992). Using a similar assay system to Last and Llewellyn (1997), these results have confirmed the result of Last and Llewellyn (1997) that radish seed extract inhibits the growth of *T. basicola* invitro. The prior work by Last and Llewellyn did not further investigate the source of the fungal growth inhibition when using radish seed extract.

The work of Terras et. al. (1992) had shown that Rs-AFP2 obtained from radish seeds was able to inhibit a range of fungal pathogens, such as *R. solani*, *V. dahliae* and *F. oxysporum*. It would have been possible to extract the Rs-AFP2 protein from radish seeds for all of the tests here. Instead, an expression system for the Rs-AFP2 protein was sought because it could potentially produce much larger yields of pure protein Cloning of the RS-AFP gene into *K. lactis* was designed to provide an expression system that would allow easy comparison using cultures of *K. lactis* containing the gene and cultures that did not contain the gene as treatments in a series of plate tests. The system that was chosen has reported yields of up to 50 mg of protein per litre of culture (NEBS) without special treatment. Genomic insertion of the Rs-AFP2 gene has been shown to protect transgenic tobacco from infection by *Alternaria longipes* (Terras et al. 1995) and tomato and canola from multiple pathogens (Parashina et al. 2000). With a view to protecting cotton against a rapidly spreading (Allen 1990; Nehl et al. 2004) and currently untreatable (Allen et al. 2008) disease threat, production and application of the protein directly to soil or as a seed coating had potential benefits compared to insertion of the Rs-AFP2 gene into the cotton genome. There are many varieties of cotton available to growers for different seasonal and environmental conditions (*2008 Variety Guide* 2008). Any treatment developed using this system could potentially be applied to all varieties within a single season. Also, as black root rot is primarily a seedling disease (Allen et al. 2008), protection is only required early in the season.

The inhibition of *T. basicola* by Rs-AFP2 was demonstrated using a growth inhibition assay using protein extract from *K. lactis* cells containing the Rs-AFP2 gene. As the protein extraction process was the same for all colonies, the high level of fungal growth in the control wells suggests that the growth inhibition observed in the remaining wells is due to the anti-fungal protein and not due to residual SDS or other chemicals from the protein extraction process. Additionally, all the yeast colonies were identical except for the presence of either the malE1 gene (controls, M1 and M2) or the Rs-AFP2 gene (31, 32, 81, 82, 83 and 84), implying that the fungal growth inhibition is due to the production of the Rs-AFP2 protein by the yeast. Increased overall fungal growth in all the supernatant samples (when compared to the protein extract samples) suggests that something in the growth medium used for the yeast was stimulating *T. basicola*. The high level of fungal growth inhibition observed in the treatment wells containing protein extract suggests that the Rs-AFP2 protein was

present in the yeast cells. It is important to note that the protein extraction process concentrated the extracts as the supernatants from a 20ml culture were used directly, while the protein extraction method uses all the cells from a 20ml culture but ends up with a final volume of approximately 200µl, a concentration of 100X and so it is not possible to directly compare the relative quantity protein inside the cell with the level of protein that was excreted.

Unfortunately, subsequent inhibition assays were not so consistent. A large degree of inconsistency in fungal growth inhibition was experienced (unpublished data), even when using identical conditions. Upon commencement of this work in 2005 we suspected that Rs-AFP2 only inhibited growth of filamentous fungi. The Rs-AFP2 gene was originally cloned in *Saccharomyces cerevisiae*, but the expression system we chose to use was a commercial expression system based on *K. lactis*. In a later search of literature it was found that Rs-AFP2 has been shown to interact with glucosylceramide in the cell membrane of fungi to inhibit growth (Thevissen et al. 2004). *S. cerevisiae* does not produce this lipid (Takakuwa et al. 2003), and as such, it is immune to the effect of Rs-AFP2. *K. lactis* does produce glucosylceramide (Takakuwa et al. 2003; Tanji et al. 2004) and so it is likely that excretion of the RS-AFP protein into the culture medium would have inhibited growth. Intracellular protein production without excretion may still have been possible, explaining the higher level of fungal growth suppression by the whole cell protein extracts.

Rs-AFP2 may still have potential as a bio-control agent for black root rot, however the strategy for its production needs to be re-evaluated in light of this information and different strains of yeast sought. One possibility is to create a knockout mutant strain of *K. lactis* that does not contain a functional gene critical to production of glucosylceramide. Such a strain has been created previously by using PCR to knock out the ceramide-glucosyltransferase gene (Saito et al. 2006). The effect of such a knockout is to decrease tolerance to alkaline growth conditions, which would not affect protein production under laboratory conditions, where growth media pH could be managed. Such a modification of the expression system would allow the existing clones, confirmed to contain the Rs-AFP2 gene to be re-used as a starting point, potentially reducing the time required to produce a functional expression system.

Acknowledgements

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5.6. References

2008 Variety Guide, 2008. Retrieved from http://www.csd.net.au/asset/send/1611/download/original/2008%20Variety%20guide.pdf

Allen, S.J. 1990, '*Thielaviopsis basicola*, a new record on cotton in Australia', *Australasian Plant Pathology,* vol. 19, no. 1, pp. 24-25.

Allen, S.J., Nehl, D.B. & Moore, N. 2008, Integrated Disease Management: Australian Cotton Cooperative Research Centre.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (eds) 1988, *Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley Interscience, New York.

Broekart, W.F., Terras, F.R.G., Cammue, B.P.A. & Osborn, R.W. 1995, 'Plant Defensins: Novel Antimicrobial Peptides as Components of the Host Defense System', *Plant Physiology,* vol. 108, pp. 1353-1358.

Climate Averages, 2009, Bureau of Meteorology. Retrieved 27/01 2009 from http://www.bom.gov.au/climate/averages/

Gao, A., Hakimi, S.M., Mittanck, C.A., Wu, Y., Woerner, B.M., Stark, D.M., Shah, D.M., Liang, J. & Rommens, C.M.T. 2000, 'Fungal pathogen protection in potato by expression of a plant defensin peptide', *Nature Biotechnology,* vol. 18, pp. 1307-1310.

Hammond-Kosack, K.M. & Jones, J.D.G. 1997, 'Plant Disease Resistance Genes', *Annual Review of Plant Physiology and Plant Molecular Biology,* vol. 48, pp. 575-607.

Harju, S., Fedosyuk, H. & Pterson, K.R. 2004, 'Rapid isolation of yeast genomic DNA: Bust n' Grab', *BMC Biotechnology,* vol. 4, no. 8

Hoffman, G., Garrison, T.R. & Dohlman, H.G. 2002, 'Analysis of RGS proteins in *Saccharomyces cerevisiae*', *Methods in Enzymology,* vol. 344, pp. 617-631.

Last, D.I. & Llewellyn, D.J. 1997, 'Antifungal proteins from seeds of Australian native plants and isolation of an antifungal peptide from *Atriplex nummularia*', *New Zealand Journal of Botany,* vol. 35, pp. 385-394.

Nehl, D.B., Allen, S.J., Mondal, A.H. & Lonergan, P.A. 2004, 'Black root rot: a pandemic in Australian cotton', *Australasian Plant Pathology,* vol. 33, pp. 87-95.

Parashina, E.V., Serdobinskii, L.A., Kalle, E.G., Lavrova, N.V., Avetisov, V.A., Lunin, V.G. & Naroditskii, B.S. 2000, 'Genetic Engineering of Oilseed Rape and Tomato Plants Expressing a Radish Defensin Gene', *Russian Journal of Plant Physiology,* vol. 47, pp. 417-423.

Saito, K., Takakuwa, N., Ohnishi, M. & Oda, Y. 2006, 'Presence of glucosylceramide in yeast and its relation to alkali tolerance of yeast', *Applied Microbiology and Biotechnology,* vol. 71, no. 4, pp. 515-521.

Takakuwa, N., Yamane, K., Kinoshita, M., Oda, Y. & Ohnishi, M. 2003, 'Distribution of cerebroside in genus *Saccharomyces* and its closely related yeasts, and cloning of cerebroside metabolism-related genes', in *Advanced research on Plant Lipids*, N. Murata, M. Yamada, I. Nishida, H. Okuyama, J. Sekiya & W. Hajime (eds), Kluwer Academic Publishers, pp. 229-232.

Tanji, M., Namimatsu, K., Kinoshita, M., Motoshima, H., Oda, Y. & Ohnishi, M. 2004, 'Content and Chemical composition of Cerebrosides in Lactose-assimilating yeasts', *Bioscience, Biotechnology, Biochemistry,* vol. 68, no. 10, pp. 2205-2208.

Terras, F.R., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., VanLeuven, F., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. 1995, 'Small cysteine-rich antifungal proteins from radish: their role in host defense', *The Plant Cell,* vol. 7, pp. 573-588.

Terras, F.R.G., Schoofs, H.M.E., De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. 1992, 'Analysis of Two Novel Classes of Plant Antifungal Proteins from Radish (*Raphanus sativus* L.) Seeds', *Journal of Biological Chemistry,* vol. 267, no. 22, pp. 15301-15309.

Thevissen, K., Terras, F.R.G. & Broekaert, W.F. 1999, 'Permeabilization of Fungal Membranes by Plant Defensins Inhibits Fungal Growth', *Appl. Environ. Microbiol.,* vol. 65, no. 12, pp. 5451-5458.

Thevissen, K., Warnecke, D.C., Francois, I.E.J.A., Leipelt, M., Heinz, E., Ott, C., Zahringer, U., Thomma, B.P.H.J., Ferket, K.K.A. & Cammue, B.P.A. 2004, 'Defensins from Insects and Plants Interact with Fungal Glucosylceramides', *J. Biol. Chem.,* vol. 279, no. 6, pp. 3900-3905.

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6 Discussion

6.1. The future for biocontrol treatments

Cotton farming in Australia is moving towards a more sustainable future, with an increasing number of growers taking part in Best Management Practice (BMP) aimed at improving farm safety and reducing environmental impact (Cotton Research and Development Corporation Annual Report 2004-05 2005). Evidence also suggests a growing demand by consumers for organic produce (BFA 2008) and the use of bio-chemicals and biologically based disease treatments could assist in the supply. Demand for organic cotton is also increasing. Sales of organic cotton worldwide was $245 million in 2001 and increased to $1.7 billion in 2007 (Organic Exchange 2007 Annual Report 2008). This increase suggests that future production of cotton will need to be supported by an increased range of biologically based products that meet organic certification.

Many biocontrol options have been proposed that are successful in the controlled conditions of a laboratory or invitro but then fail to deliver when transferred to soil systems or the field (Lindow 1988a; Reddy et al. 1994). It is possible that there are more that were never published. This study has investigated three potential systems for suppressing *T. basicola*, but throughout, the end goal has been to provide a solution that Australian cotton growers can use to improve their crop yield and maintain their position as one of the most efficient cotton industries in the world (International Cotton Advisory Committee 2007).

There are a number of products on the market that prove biologically based treatments can be commercially viable. Sentinel™ (Agrimm Technologies) is a new product registered in New Zealand based on a Trichoderma species. It is sold in powder form and is mixed with water before spray application to protect grapes and tomatoes from Botrytis mould and stem rot respectively. The same company also supplies Plantmate™ bio-inoculant which it sells internationally. These products are organic certified, meaning that farmers who are trying to meet certification requirements can use these products without jeopardising the premium price they may be able to obtain for their organic products. Biocontrol of

diseases is field that has attracted interest over a number of years. Fravel (2005) reviewed biocontrol for plant diseases over nearly 20 years and found that the diversity of biocontrol options being tested increased (to 2004), and that while the first product was registered in 1979, 65% of registered products had been registered between 1994 and 2004.

This work has investigated three potential systems using soil bacteria, soil amendments and an antifungal protein that could potentially meet the criteria of large scale application and ready availability. Two of these systems also meet the criteria of being organic, based on either whole organisms (application of soil bacteria), plant or plant by-products (soil amendments). Although the protein expression system would not be considered organic, it may be more acceptable in the community than a synthetic chemical as it is a 'natural' product.

6.2. Inoculation of plants with bacterial species can protect plants from disease

The study presented here has shown that *A. brasilense* can be used in the field as a bio-inoculant to reduce the incidence of *T. basicola* induced root necrosis on cotton seedlings, thought the mechanism was not determined. This adds to the research of Fayez and Daw (1987) who found that cotton roots could be inoculated with *A. brasilense* by dipping in a suspension of bacteria prior to planting to increase plant height and nitrogen uptake. *A. brasilense* is commonly known as a plant growth promoting (PGP) species (Okon & Labandera-Gonzalez 1994). In a review in 2004 a number of microbial species are identified including *Azospirillum*, *Clostridium*, *Rhizobium* and *Acetobacter* that promote plant growth through fixation of nitrogen (Kennedy et al. 2004). Recent research has suggested that PGP strains (including *A. brasilense*) can also induce host response against pathogenic species (Ramos Solano et al. 2008). Multiple PGP *Bacillus* strains have also been identified that protect plants against disease even though they exhibit no direct antibiosis invitro (Jetiyanon & Kloepper 2002). This study did not test for antibiosis by *A. brasilense* against *T. basicola* invitro. This may be a possible mechanism for suppression of *T. basicola* as *Azospirillum* sp. have been shown to be capable of producing antifungal compounds and siderophores in association with cotton (Pandey & Kumar 1989), although invitro tests may not

reveal if this is the cause of disease suppression as some researchers have reported a lack of correlation between invitro testing for antibiosis with activity against a disease in a soil system (Reddy et al. 1994) or in conjunction with plant tissue (Lindow 1988a). Another possible mechanism is through production of indole-3-acetic acid (IAA). *A. brasilense* produces IAA (Tien et al. 1979) and exogenous IAA production by bacteria has been implicated in a faster plant response to fungal pathogens (Fernandez-Falcon et al. 2003).

Strains of *P. agglomerans* have been shown to inhibit post-harvest *Penicillium* mold in oranges through an unknown mechanism (Teixidó et al. 2001) and reduce the incidence of fireblight in apples, partially via antibiosis (Stockwell et al. 2002) and bacteria in the Microbacterium genus have been implicated in suppression of *Pythium* damping off (Postma et al. 2005) and bacterial blight (Fukui et al. 1999) (both through undefined mechanisms). With the presence of *E. acetylicum*, these three species (PEM) have been shown to inhibit *R. solani* in wheat growing soil (Barnett et al. 2006). Some strains have also been shown to cause disease rather than inhibit it. *P. agglomerans* has been found to cause formation of galls in Gypsophilae (an ornamental plant) preventing root development in cuttings (Brown 1932; Manulis & Barash 2003). Before completing field trials, pot trials confirmed that there was no increase in black root rot symptoms and that no additional disease symptoms were apparent when the strains under investigation were applied to soil as a planting treatment.

This study has shown that strains from these three species can inhibit *T. basicola* in sterile pot systems. Individually, these species did not exhibit any significant suppression of directional fungal growth either during invitro plate tests for antibiosis or in the presence of cotton. When required to compete/interact with each other, or with another non-biocontrol species (*E. coli* DH5α) the PEM species inhibited fungal hyphal growth towards cotton. It was not fully confirmed if this was through antibiosis or some other mechanism blocking the signalling between *T. basicola* and the cotton plant. Nevertheless, there is evidence to suggest that it was not due to blocking of active signalling during pathogen-plant interaction, as PEM also blocked the growth of the fungus towards cotton root extract in the invitro directional growth tests. This does not eliminate blocking of a passive

signal. Directional growth of fungal hyphae towards a host has been shown to be important for some species. Although not pathogenic, Mycorrhiza are strongly attracted to plant roots (Vierheilig et al. 1998) and root exudates stimulate a change in direction of hyphal growth towards roots (Giovannetti et al. 1993). In work in this laboratory, *T. basicola* has also been shown to exhibit a significant directional growth preference towards host plants (Pereg & Al-Jaiidi, unpublished). Additionally, in soil, *T. basicola* requires living tissue to reproduce (Hood & Shew 1997b) and so reduced hyphal growth towards living root tissue will decrease the possibility of *T. basicola* producing new spores, potentially reducing inoculum load in the soil.

Despite an apparent trend towards lower mean disease levels, neither *A. brasilense* or PEM significantly inhibited black root rot symptoms at several field trial sites. A combination of *A. brasilense* and PEM may prove to be effective at more sites. *Azospirillum* species are common in Australian soils (Sung & New 1998) and the PEM species tested were isolated from Australian soils. This may prove to be important when suggesting these species as a sustainable treatment as they are local species and have not been introduced to Australia. Additionally, only one (high) level of inoculum was tested. Further testing using different application rates may prove beneficial.

6.3. Rs-AFP2 inhibits T. basicola but efficacy as a field inoculant has not been established

It was not possible to obtain the strain with the Rs-AFP2 gene already cloned (as published, Terras et al. 1992) so it was intended that a new system be developed. As it was planned to produce large quantities of the protein for application to the soil in pot trials, a commercial yeast, *K. lactis* was chosen to host the Rs-AFP2 gene. The Rs-AFP2 gene was successfully inserted into the *K. lactis* genome and in one test a high degree of inhibition of *T. basicola* was seen, but further testing gave inconsistent results. Later searches of the published literature revealed the potential cause of this inconsistency.

Rs-AFP2 reduces fungal growth by permeabilising the cell membrane (Thevissen et al. 1999) through an interaction with a specific phospholipid (glucosylceramide) contained in the cell wall of many fungi (Thevissen et al. 2004). Much of the published literature on Rs-AFP2 had tested its activity against a range of filamentous fungi. For example, (Terras et al. 1992) tested the proteins activity against 20 different species, all filamentous. Later work confirmed the inability of Rs-AFP2 to induce membrane permeability in *S. cerevisiae* (Thevissen et al. 1999). When the work in this study was commenced, we did not have access to the 2004 study of Thevissen et al. (Thevissen et al. 2004) and (incorrectly) reasoned that as Rs-AFP2 inhibited many different filamentous fungi and did not inhibit *S. cerevisiae*, that other similar yeasts would not be inhibited either. The 2004 study highlights that this was an incorrect view and that the *K. lactis* strain used was not a suitable host for production of Rs-AFP2. Production of the protein without excretion was still possible (and appears to have happened in this case) as there would then be no interaction with the cell membrane, but this was not the intention of using the *K. lactis* vector, which was designed for protein excretion to minimise processing during protein purification.

Rs-AFP2 may still have potential as an agent against *T. basicola* in the field as it has many properties that would make it suitable for field application. Given an appropriate production system, large quantities of protein could be produced. Although the strain used was not a suitable host, the *K. lactis* system used in this study has been known to yield 50mg of protein per litre without using specialised high density fermentation techniques (NEBS). It has previously been shown that it is possible to create a mutant strain of *K. lactis* that does not contain a functional gene critical to production of glucosylceramide (Saito et al. 2006). Applying this research to the strain of *K. lactis* used in this study may provide a method of producing Rs-AFP2 with minimal modification to the current system. Rs-AFP2 has been shown in vitro to be toxic to some fungi at levels as low as 2μg/ml (Terras et al. 1992) so with a suitable host, the production of large quantities of an antifungal treatment based on Rs-AFP2 would be technically feasible. The protein is also quite stable, surviving heating to 100°C (Terras et al. 1992). Heat stability would be important as daytime temperatures in cotton growing regions can exceed 40°C

(*Climate Averages* 2009) and any product produced would need to withstand on farm storage prior to application.

The Rs-AFP2 protein has been used to protect crops from disease through genetic modification. Tomato and canola plants have been engineered with the Rs-AFP2 gene to defend against multiple pathogens (Parashina et al. 2000). It was also suggested in a 2000 patent application that a modified gene based on a radish defence protein (Rs-AFP2) could be inserted into plant genomes (including cotton) to inhibit disease (Posthuma et al. 2001) but eight years later, no cotton varieties have been brought to market with this trait, suggesting that introduction of a resistant strain may still be some time off, highlighting the need for other solutions in the mean time. As previously discussed (1.2.1), use of GM technologies can also have issues of acceptance and regulatory approval. As a specific example from cotton, Roundup Ready® cotton was approved in parts of Australia in 2002 (OGTR 2002), but another four years of trials were required before approval for planting north of the 22nd parallel was granted in 2006 (OGTR 2006).

Although this work did not develop a suitable system for reducing black root rot in this case, Rs-AFP2 or proteins with similar properties should be sought and tested with the view to developing a treatment for this disease.

6.4. Soil amendments can inhibit disease but three suggested amendments are not recommended for field application

It has been shown that in relation to black root rot in cotton, the single biggest factor in disease severity in a given season is how many host (cotton) crops have previously been planted (Nehl et al. 2004). As infection and production of spores in the soil requires living tissue (Hood & Shew 1997b), soil amendments that can induce the germination of spores in the absence of a host plant could reduce fungal pathogen load.

A pilot study was conducted to determine if any of the three selected amendments, yeast extract, zein or soy flour had potential for application in field trials. After comparing the ability of non-amended soil infested with *T. basicola* spores to infect

cotton seedlings with amended soil, the ability of these selected amendments to suppress disease demonstrated in the 1969 study was not replicated. This was not entirely unexpected, as the effectiveness of organic soil amendments have been reported to vary depending on soil type or even moisture conditions (Lazarovits 2001; Lazarovits et al. 2001). As previously stated, the goal of this study was to find agents that could be used to suppress black root rot in field applications under Australian conditions. As it was not demonstrated that any of these three agents reduced infection rates in pot trials using soil obtained from cotton growing fields, field trials were not conducted.

6.5. Conclusions:

The rapid increase in demand for organic products and increased industry support for sustainable agriculture is likely to continue and crop producers will need to be supported in their efforts to reduce chemicals by the development of products that help them to meet the rising demand. There are products available proving that biologically based disease treatments can be commercially viable. The bacterial treatments presented here were successfully in reducing disease in controlled conditions, but not under field conditions. Further research is required to determine if there is an effect on yield.

The antifungal protein Rs-AFP2 was confirmed to inhibit *T. basicola* invitro but it has not been determined if this can be translated into an effective field bio-fungicide. A better production system for this protein is needed so testing can be completed. The selected soil amendments tested did not yield any consistent suppression of black root rot in pot trials and so were not further tested in the field. Although these two systems did not lead to successful treatment of black root rot symptoms in the field, this type of research is still important in the search for effective treatments.

6.6. Complete reference list

2008 Variety Guide, 2008. Retrieved from http://www.csd.net.au/asset/send/1611/download/original/2008%20Variety%20guide.pdf

Allen, S.J. 1990, '*Thielaviopsis basicola*, a new record on cotton in Australia', *Australasian Plant Pathology,* vol. 19, no. 1, pp. 24-25.

Allen, S.J., Nehl, D.B. & Moore, N. 'Appendix 1, Variey Resistance Rankings', in *Integrated Disease Management Manual*, Australian Cotton Cooperative Research Centre

Allen, S.J., Nehl, D.B. & Moore, N. *Integrated Disease Management*, Australian Cotton Cooperative Research Centre. Retrieved from http://www.cottoncrc.org.au/content/Industry/Publications/DiseaseMicrobiology/Cotton_IDM_Guidelines.aspx

Allen, S.J., Nehl, D.B. & Moore, N. 2008, Integrated Disease Management: Australian Cotton Cooperative Research Centre.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (eds) 1988, *Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley Interscience, New York.

Barnett, S.J., Roget, D.K. & Ryder, M.H. 2006, 'Suppression of *Rhizoctonia solani* AG-8 induced disease on wheat by the interaction between *Pantoea*, *Exiguobacterium*, and *Microbacteria*', *Australian Journal of Soil Research,* vol. 44, no. 4, pp. 331-342.

Bashan, Y. 1998, 'Inoculants of Plant Growth-promoting Bacteria for use in Agriculture', *Biotechnology Advances,* vol. 16, no. 4, pp. 729-770.

Bashan, Y. & de-Bashan, L.E. 2002, 'Protection of Tomato Seedlings against Infection by Pseudomonas syringae pv. Tomato by Using the Plant Growth-Promoting Bacterium *Azospirillum brasilense*', *Applied and Environmental Microbiology,* vol. 68, no. 6, pp. 2637-2643.

Bates, G. & Rothrock, C.S. 2005, 'Potential use of high glucosinolate Indian mustard and canola for suppression of soilborne pathogens', *Phytopathology,* vol. 95, no. 6, suppl., p. S7.

BFA 2008, (updated 22/7), *New research reveals over 80% growth in organic farm gate sales since 2004 despite widespread drought*, Biological Farmers of Australia Retrieved from http://www.bfa.com.au/index.asp?Sec_ID=259

Broekaert, W.F., Terras, F.R. & Cammue, B.P.A. 2000, 'Induced and preformed antimicrobial proteins', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Fraser & L.C. Van Loon (eds), Kluwer Academic Publishers, Dordrecht

Broekart, W.F., Terras, F.R.G., Cammue, B.P.A. & Osborn, R.W. 1995, 'Plant Defensins: Novel Antimicrobial Peptides as Components of the Host Defense System', *Plant Physiology,* vol. 108, pp. 1353-1358.

Brown, N.A. 1932, 'A gall similar to crown gall produced on Gypsophila by a new bacterium', *Jounal of Agricultural Research,* vol. 48, pp. 1099-1112.

Candole, B.L. & Rothrock, C.S. 1997, 'Characterisation oif the Suppressiveness of Hairy Vetch-Ammended soils to *Thielaviopsis basicola*', *Phytopathology,* vol. 87, no. 2, pp. 197-202.

Candole, B.L. & Rothrock, C.S. 1998, 'Using marked strains to assess the effect of hairy vetch amendment on the inoculum densities of *Thielaviopsis basicola, Pythium ultimum* and *Rhizoctonia solani*', *Soil Biol. Biochem.,* vol. 30, no. 4, pp. 443-448.

Chisholm, S.T., Coaker, G., Day, B. & Staskawicz, B.J. 2006, 'Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response', *Cell,* vol. 124, no. 4, pp. 803-814.

Christou, T. 1962, 'Penetration and host-parasite relationships of *Thielaviopsis basicola* on the bean plant', *Phytopathology,* vol. 52, pp. 194-198.

Climate Averages, 2009, Bureau of Meteorology. Retrieved 27/01 2009 from http://www.bom.gov.au/climate/averages/

Colson-Hanks, E.S. & Deverall, B.J. 2000, 'Effect of 2,6-dichloroisonicotinic acid, its formulation materials and benzothiadiazole on systemic resistance to alternaria leaf spot in cotton ', *Plant Pathology,* vol. 49, pp. 171-178.

Conant, G.H. 1927, 'Histological studies of resistance in tobacco to *Thielavia basicola*', *American Journal of Botany,* vol. 14, no. 8, pp. 457-480.

Cotton Australia. 2008, 'Australian Cotton History', in *Fact Sheet Book*, Cotton Australia, Mascot, NSW

Cotton Research and Development Corporation Annual Report 2004. 2005. Narrabri: Cotton Research and Development Corporation.

Cotton Research and Development Corporation Annual Report 2004-05. 2005. Narrabri: Cotton Research and Development Corporation.

Cotton-Australia 2009, *Facts and Figures: General; Cotton in Australia*. Retrieved from http://www.cottonaustralia.com.au/facts/factsandfigures.aspx?id=18

Current Drought Situation, 2008. Retrieved 16/1 2008 from http://www.dpi.nsw.gov.au/agriculture/emergency/drought/situation/drought-maps/drought-maps

Delevecchio, V.G., Corbaz, R. & Turian, G. 1969, 'An ultrastructural study of the hyphae, endoconidida and chlamydospores of *Thielaviopsis basicola*', *Journal of General Microbiology,* vol. 58, pp. 23-27.

Disease Ranks, 2008, Cotton Seed Distributors. Retrieved from http://www.csd.net.au/page/show/21091/

Edreva, A. 2004, 'A novel strategy for plant protection: Induced resistance', *Journal of Cell and Molecular Biology,* vol. 3, pp. 61-69.

El-Wakiel, N.E. & El-Sebai, T.N. 2008, 'Role of biofertilizer on faba bean growth, yield, and its effect on bean aphid and the associated predators ', *Archives of Phytopathology and Plant Protection*

Fayez, M. & Daw, Z.Y. 1987, 'Effect of inoculation with different strains of *Azospirillum brasilense* on cotton (*Gossypium barbadense*)', *Biology and Fertility of Soils,* vol. 32, pp. 47-51.

Fernandez-Falcon, M., Borges, A.A. & Borges-Perez, A. 2003, 'Induced resistance to Fusarium wilt of banana by exogenous applications of indole acetic acid', *Phytoprotection,* vol. 84, pp. 149-153.

Francis, J. 2006, 'GM Crops, the Precautionary Principle and Canola: Do the Exceptions Prove the Rule? ' *Australian Political Studies Association Conference*

Frazel, D.R. 2005, 'Commercialization and Implementation of Biocontrol', *Annual Review of Phytopathology*, vol. 45, pp. 337-359.

Fukui, R., Fukui, H. & Alvarez, A.M. 1999, 'Suppression of Bacterial Blight by a Bacterial Community Isolated from the Guttation Fluids of Anthuriums', *Applied and Environmental Microbiology,* vol. 65, no. 3, pp. 1020-1028.

Fulchieri, M. & Frioni, L. 1994, 'Azospirillum inoculation on maize (Zea mays): effect on yield in a field experiment in central argentina', *Soil Biology and Biochemistry,* vol. 26, no. 7, pp. 921-923.

Gao, A., Hakimi, S.M., Mittanck, C.A., Wu, Y., Woerner, B.M., Stark, D.M., Shah, D.M., Liang, J. & Rommens, C.M.T. 2000, 'Fungal pathogen protection in potato by expression of a plant defensin peptide', *Nature Biotechnology,* vol. 18, pp. 1307-1310.

Gayed, S.K. 1969, 'The relation between tobacco leaf and root necrosis induced by *Thielaviopsis basicola* resistance to black root rot', *Phytopathology,* vol. 59, pp. 1596-1600.

Giovannetti, M., Sbrana, C., Avio, L., Citernesi, A.S. & Logi, C. 1993, 'Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages', *New Phytologist,* vol. 125, no. 3, pp. 587-593.

Hake, K., DeVay, J., Kerby, P., Garber, R. & Chrisco, L. 1985, 'Cultural control of cotton black root rot', *Beltwide Cotton Production Research Conference*, pp. 25-26.

Hammond-Kosack, K.M. & Jones, J.D.G. 1997, 'Plant Disease Resistance Genes', *Annual Review of Plant Physiology and Plant Molecular Biology,* vol. 48, pp. 575-607.

Harju, S., Fedosyuk, H. & Pterson, K.R. 2004, 'Rapid isolation of yeast genomic DNA: Bust n' Grab', *BMC Biotechnology,* vol. 4, no. 8

Harrison, U.J. & Shew, H.D. 2001, 'Effects of soil pH and nitrogen fertility on the population dynamics of *Thielaviopsis basicola*', *Plant and Soil,* vol. 228, pp. 147-155.

Harwood, R.R. 1990, 'A history of sustainable agriculture', in *Sustainable agricultural systems*, C.A. Edwards, R. Lal, P. Madden, H.R. Miller & G. House (eds), CRC Press, pp. 3-19.

Hoffman, G., Garrison, T.R. & Dohlman, H.G. 2002, 'Analysis of RGS proteins in *Saccharomyces cerevisiae*', *Methods in Enzymology,* vol. 344, pp. 617-631.

Hoitink, H.A.J. & Fahy, P.C. 1986, 'Basis for the control of soilborne plant pathogens with composts', *Annual Review of Phytopathology,* vol. 24, pp. 93-114.

Hood, M.E. & Shew, H.D. 1997a, 'Initial cellular interactions between *Thielaviopsis basicola* and tobacco root hairs', *Phytopathology,* vol. 87, no. 3, pp. 228-235.

Hood, M.E. & Shew, H.D. 1997b, 'Reassesment of the role of saprophytic activity in the ecology of *Thielaviopsis basicola*', *Phytopathology,* vol. 87, no. 12, pp. 1214-1219.

Huang, H.C. & Patrick, Z.A. 1971, 'Variability of *Thielaviopsis basicola* in culture', *Canadian Journal of Botany,* vol. 49, no. 6, pp. 1041-1047.

Inglis, D.A. & Cook, R.J. 1986, 'Persistence of Chlamydospores of *Fusarium culmonorum* in Wheat Field Soils of Eastern Washington', *Phytopathology,* vol. 76, no. 11, pp. 1205-1208.

International Cotton Advisory Committee. 2007, Cotton: World statistics: Secretariat of the International Cotton Advisory Committee.

Jabs, T. & Slusarenko, A.J. 2000, 'The hypersensitive response', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Fraser & L.C. Van Loon (eds), Kluwer Academic Publishers, Dordrecht

Jetiyanon, K. & Kloepper, J.W. 2002, 'Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases', *Biological Control,* vol. 24, pp. 285-291.

Johnson, J. 1916, 'Host plants of *Thielavia basicola*', *Journal of Agricultural Research,* vol. 7, no. 6, pp. 289-300.

Kennedy, I.R., Choudhury, A.T.M.A. & Kecskes, M.L. 2004, 'Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited?' *Soil Biology and Biochemistry,* vol. 36, no. 8, pp. 1229-1244.

King, C.J. & Presley, J.T. 1942, 'A root rot of cotton caused by *Thielaviopsis basicola*', *Phytopathology,* vol. 32, pp. 752-761.

Knudsen, I.M.B., Hockenhull, J., Funck Jensen, D., Gerhardson, B., Hokeberg, M., Tahvonen, R., Teperi, E., Sundheim, L. & Henriksen, B. 1997, 'Selection of biological control agents for controlling soil and seed-borne diseases in the field', *European Journal of Plant Pathology,* vol. 103, pp. 775-784.

Kristyanne, E.S., Kim, K.S. & Stewart, J.M. 1997, 'Magainin effects on the ultrastructure of five plant pathogens', *Mycologia,* vol. 89, no. 3, pp. 353-360.

Lamb, C. & Dixon, R.A. 1997, 'The oxidadtive burst in plant disease resistance', *Annual Review of Plant Physiology and Plant Molecular Biology,* vol. 48, no. 1, pp. 251-275.

Last, D.I. & Llewellyn, D.J. 1997, 'Antifungal proteins from seeds of Australian native plants and isolation of an antifungal peptide from *Atriplex nummularia*', *New Zealand Journal of Botany,* vol. 35, pp. 385-394.

Lazarovits, G. 2001, 'Management of soil-borne plant pathogens with organic soil amendments: a disease control strategy salvaged from the past ', *Canadian Journal of Plant Pathology,* vol. 23, pp. 1-7.

Lazarovits, G., Tenuta, M. & Conn, K.L. 2001, 'Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops', *Australasian Plant Pathology,* vol. 30, no. 2, pp. 111-117.

Leeman, M., Pelt, J.A.v., Ouden, F.M.d., Heinsbroek, M., Bakker, P.A.H.M. & Schoippers, B. 1995, 'Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of Pseudomonas fluorescens', *Phytopathology,* vol. 85, no. 9, pp. 1021-1027.

Lindow, S.E. 1988a, 'Lack of correlation of In vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria', *Ecology and Epidemiology,* vol. 78, pp. 444-450.

Liu, L., Kloepper, J.W. & Tuzun, S. 1995, 'Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria', *Phytopathology,* vol. 85, no. 8, pp. 843-847.

Lopez, L., Morales, G., Wolff, M. & Lowenberger, C. 2003, 'Isolation and characterization of a novel insect defensin from *Rhodnius prolixus*, a vector of Chagas disease', *Insect Biochemistry and Molecular Biology,* vol. 33, pp. 439-447.

Lucas, G.B. 1955, 'The cardinal temperatures and pH response of *Thielaviopsis basicola*', *Mycologia,* vol. 47, pp. 793-798.

Manulis, S. & Barash, I. 2003, 'Pantoea agglomerans pvs. gypsophilae and betae, recently evolved pathogens?' *Molecular Plant Pathology,* vol. 4, no. 5, pp. 307-314.

Mathre, D.E. & Ravenscroft, A.V. 1966, 'Physiology of germination of chlamydospores and endoconidia of *Thielaviopsis basicola*', *Phytopathology,* vol. 56, pp. 337-342.

Mathre, D.E., Ravenscroft, A.V. & Garber, R.H. 1966, 'The role of *Thielaviopsis basicola* as a primary cause of yield reduction in cotton in California', *Phytopathology,* vol. 56, pp. 1119-1212.

Mauk, P.A. & Hine, R.B. 1988, 'Infection, colonization of *Gossypium hirsutum* and *G. barbadense*, and development of black root rot caused by *Thielaviopsis basicola*', *Phytopathology,* vol. 78, no. 12, pp. 1662-1667.

Metraux, J. 2001, 'Systemic acquired resistance and salicylic acid: current state of knowledge', *European Journal of Plant Pathology,* vol. 107, pp. 13-18.

Meyer, J.R. & Shew, H.D. 1991a, 'Development of black root rot in burley tobacco as influenced by inoculum density of *Thielaviopsis basicola*, host resistance, and soil chemistry', *Plant Disease,* vol. 75, pp. 601-605.

Meyer, J.R. & Shew, H.D. 1991b, 'Soils suppressive to black root rot of burley tobacco, caused by *Thielaviopsis basicola*', *Phytopathology,* vol. 81, no. 9, pp. 946-954.

Meyer, J.R., Shew, H.D. & Harrison, U.J. 1994, 'Inhibition of germination and growth of *Thielaviopsis basicola* by aluminum', *Phytopathology,* vol. 84, pp. 598-602.

Mims, C.W., Copes, W.E. & Richardson, E.A. 2000, 'Ultrastructure of the penetration and infection of pansy roots by *Thielaviopsis basicola*', *Phytopathology,* vol. 90, no. 8, pp. 843-850.

Moerschbacher, B. & Mendgen, K. 2000, 'Structural aspects of defense', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Frase & L.C. van Loon (eds), Kluwer Academic Publishers, Dordrecht

Mondal, A.H., Nehl, D.B. & Allen, S.J. 2004, 'First report of *Thielaviopsis basicola* on soybean in Australia', *Australasian Plant Pathology,* vol. 33, pp. 451-452.

Mondal, A.H., Nehl, D.B. & Allen, S.J. 2005, 'Acibenzolar-S-methyl induces systemic resistance in cotton against black root rot casued by *Thielaviopsis basicola*', *Australasian Plant Pathology,* vol. 34, pp. 499-507.

Mondal, A.H., Nehl, D.B. & Deverall, B.J. 2000, 'Induced resistance can protect cotton and legumes from black root rot', *10th Australian Cotton Conference*

Nag Raj, T.R. & Kendrick, B. 1975, *A monograph of Chalara and allied genera*, Wilfred Lanvier Press, Waterloo, Ontario, Canada.

National Standard for Organic and Bio-dymanic Produce, 2005. Retrieved from http://www.ofa.org.au/papers/2005%20Draft%20NATIONAL%20STANDARD.pdf

Nehl, D.B., Allen, S.J., Lonergan, P.A., McNamara, G., Swan, L. & Smith, L.J. 2007, *Cotton Pathology 2006-2007*. Retrieved from http://www.cottoncrc.org.au/content/Industry/Publications/Disease__Microbiology/Disease_Surveys/2006_-_2007.aspx

Nehl, D.B., Allen, S.J., Mondal, A.H. & Lonergan, P.A. 2004, 'Black root rot: a pandemic in Australian cotton', *Australasian Plant Pathology,* vol. 33, pp. 87-95.

Nehl, D.B., Mondal, A.H. & Allen, S.J. (eds). 2000, *Managing black root rot,* Brisbane, Australia.

Nguyen, H.T., Deaker, R., Kennedy, I.R. & Roughley, R.J. 2003, 'The Positive Yield Response of Field-Grown Rice to Inoculation with a Multi-Strain Biofertiliser in the Hanoi Area, Vietnam ', *Symbiosis,* vol. 35, no. 1-3, pp. 231-245.

Nustorova, M., Braikova, D., Gousterova, A., Vasileva-Tonkova, E. & Nedkov, P. 2006, 'Chemical, microbiological and plant analysis of soil fertilized with alkaline hydrolysate of sheep's wool waste', *World Journal of Microbiology and Biotechnology,* vol. 22, no. 4, pp. 383-390.

O'Brien, R.G. & Davis, R.D. 1994, 'Lettuce black root rot - a disease caused by *Chalara elegans* ', *Australasian Plant Pathology,* vol. 23, pp. 106-111.

O'Sullivan, D.J. & O'Gara, F. 1992, 'Traits of fluorescent Pseudomonas spp. involved in suppression of plant root pathogens', *Microbiology and Molecular Biology Reviews,* vol. 56, no. 4, pp. 662-676.

OGTR 2002, *DIR 012/2002 - Commercial release of Bollgard II® cotton*. Retrieved from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir012-2002

OGTR 2006, *Decision on issuing a licence for application DIR 066/2006; commercial release of five herbicide tolerant and/or insect resistant GM cotton lines in northern Australia*. Retrieved from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir066-2006

OGTR 2008, (updated 22/08/2008), *Legislation; The Regulatory Scheme for Genetically Modified Organisms*. Retrieved 1/2/2009 from http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dirclass-2

Okon, Y. & Labandera-Gonzalez, C.A. 1994, 'Agronomic applications of *Azospirillum*: An evaluation of 20 years of worldwide field inoculation', *Soil Biol. Biochem.,* vol. 26, no. 2, pp. 1591-1601.

Organic Exchange 2007 Annual Report, 2008. Retrieved from http://www.organicexchange.org/Documents/annual07.pdf

Owen, K.J., Green, C.D. & Deverall, B.J. 2002, 'A benzothiadiazole applied to foliage reduces development and egg deposition by *Meloidogyne* spp. in glasshouse-grown grapevine roots', *Australasian Plant Pathology,* vol. 31, pp. 47-53.

Oyarzun, P.J., Gerlagh, M. & Zadoks, J.C. 1998, 'Factors associated with soil receptivity to some fungal root rot pathogens of peas', *Applied Soil Ecology,* vol. 10, pp. 151-169.

Paclt, J. 1960, 'Correct Name for the Type of Thielavia Zopf', *Taxonomy,* vol. 9, no. 1, pp. 26-27.

Pandey, A. & Kumar, S. 1989, 'Potential of *Azetobacters* and Azospirilla as biofertilizers for upland agriculture: a review', *Journal of Scientific and Industrial Research,* vol. 48, pp. 134-144.

Papavizas, G.C. & Adams, P.B. 1969, 'Survival of root-infecting fungi in soil XII. Germination and survival of endoconidia and chlamydospores of *Thielaviopsis basicola* in fallow soil and in soil adjacent to germinating bean seed', *Phytopathology,* vol. 59, pp. 371-378.

Papavizas, G.C. & Kovacs Jr, M.F. 1972, 'Stimulation of Spore Germination of *Thielaviopsis basicola* by Fatty Acids from Rhizosphere Soil', *Phytopathology,* vol. 62, pp. 688-694.

Parashina, E.V., Serdobinskii, L.A., Kalle, E.G., Lavrova, N.V., Avetisov, V.A., Lunin, V.G. & Naroditskii, B.S. 2000, 'Genetic Engineering of Oilseed Rape and Tomato Plants Expressing a Radish Defensin Gene', *Russian Journal of Plant Physiology,* vol. 47, pp. 417-423.

Paulin-Mahady, A.E., Harrington, T.C. & McNew, D. 2002, 'Phylogenetic and taxonomic evaluation of *Chalara*, *Chalaropsis*, and *Thielaviopsis*

anamorphs associated with *Ceratocyctis*', *Mycologia,* vol. 94, no. 1, pp. 62-72.

Paulitz, T., Nowak-Thompson, B., Gamard, P., Tsang, E. & Loper, J. 2000, 'A novel antifungal furanone from *Pseudomonas aureofaciens*, a biocontrol agent of fungal plant pathogens', *Journal of Chemical Ecology,* vol. 26, no. 6, pp. 1515-1524.

Perlak, F.J., Oppenhuizen, M., Gustafson, K., Voth, R., Sivasupraniam, S., Heering, D., Carey, B., Ihrig, R.A. & Roberts, J.K. 2007, 'Development and commercial use of Bollgard cotton in the USA - early promises versus todays reality', *The Plant Journal,* vol. 27, no. 6, pp. 489-501.

Posthuma, G.A., Schapper, W.M.M., Sijtsma, L., Van Amerongen, A., Fant, F. & Borremans, F.A.M. 2000, *Plant Defensin Variants*. Retrieved 26/1/2009 from http://www.wipo.int/pctdb/en/wo.jsp?IA=GB2000002941&DISPLAY=STATUS

Postma, J., Geraats, B.P.J., Pastoor, R. & van Elsas, J.D. 2005, 'Characterization of the Microbial Community Involved in the Suppression of Pythium aphanidermatum in Cucumber Grown on Rockwool', *Phytopathology,* vol. 95, no. 7, p. 808.

Pray, C.E., Huang, J., Hu, R. & Rozelle, S. 2002, 'Five years of Bt cotton in China - the benefits continue', *The Plant Journal,* vol. 31, no. 4, pp. 423-430.

Punja, Z.K. 1993, 'Influence of culture conditions on mycelial growth and phialospore production and germination in *Chalara elegans*', *Canadian Journal of Botany,* vol. 71, pp. 447-456.

Punja, Z.K. & Sun, L. 1999, 'Morphological and molecular characterization of *Chalara elegans (Thielaviopsis basicola)*, cause of black root rot on diverse plant species', *Canadian Journal of Botany,* vol. 77, pp. 1801-1812.

Pyke, B.A. (ed). 2007, *The impact of high adoption of Bollgard®II cotton on pest management in Australia,* Lubbock, TX, USA.

Raaijmakers, J.M., Vlami, M. & de Souza, J.T. 2002, 'Antibiotic production by bacterial biocontrol agents', *Antonie van Leeuwenhoeck,* vol. 81, pp. 537-547.

Ramos Solano, B., Barriuso Maicas, J., Pereyra de la Iglesia, M.T., Domenech, J. & Gutierrez Manero, F.J. 2008, 'Systemic Disease Protection Elicited by Plant Growth Promoting Rhizobacteria Strains: Relationship Between Metabolic Responses, Systemic Disease Protection, and Biotic Elicitors', *Phytopathology,* vol. 98, no. 4, pp. 451-457.

Reddy, M.S., Hynes, R.K. & Lazarovits, G. 1994, 'Relationship between in vitro growth inhibition of pathogens and suppression of preemergence damping-off and postemergence root rot of white bean seedlings in the greenhouse by bacteria ', *Canadian Journal of Microbiology,* vol. 40, no. 2, pp. 113-119.

Rengel, Z. & Marschner, P. 2005, 'Nutrient availability and management in the rhizosphere: exploiting genotypic differences', *New Phytologist,* vol. 168, no. 2, pp. 305-312.

Riggs, W. & Mims, C.W. 2000, 'Ultrastructure of chlamydospore development in the plant pathogenic fungus *Thielaviopsis basicola*', *Mycologia,* vol. 92, pp. 123-129.

Romero, A.M., Correa, O.S., Moccia, S. & Rivas, J.G. 2003, 'Effect of Azospirillum-mediated plant growth promotion on the development of bacterial diseases on fresh-market and cherry tomato ', *Journal of Applied Microbiology,* vol. 95, no. 4, pp. 832-838.

Roper, M.M. & Ladha, J.K. 1995, 'Biological N2 fixation by heterotrophic and phototrophic bacteria in association with straw', *Plant and Soil,* vol. 174, no. 1-2, pp. 211-224.

Rothrock, C.S. 1992, 'Influence of Soil Temperature, Water, and Texture on *Thielaviopsis basicola* and Black Root Rot of Cotton', *Phytopathology,* vol. 82, no. 10, pp. 1202-1206.

Rothrock, C.S., Kirkpatrick, T.L., Frans, R.E. & Scott, H.D. 1995, 'The influence of winter legume cover crops on soilborne plant pathogens and cotton seedling diseases', *Plant Disease,* vol. 79, pp. 167-171.

Saito, K., Takakuwa, N., Ohnishi, M. & Oda, Y. 2006, 'Presence of glucosylceramide in yeast and its relation to alkali tolerance of yeast', *Applied Microbiology and Biotechnology,* vol. 71, no. 4, pp. 515-521.

Selitrennikoff, C.P. 2001, 'Antifungal proteins', *Applied and Environmental Microbiology,* vol. 67, no. 7, pp. 2883-2894.

Selvakumar, G., Joshi, P., Nazim, S., Mishra, P., Kundu, S. & Gupta, H. 2009, 'Exiguobacterium acetylicum strain 1P (MTCC 8707) a novel bacterial antagonist from the North Western Indian Himalayas', *World Journal of Microbiology and Biotechnology,* vol. 25, no. 1, pp. 131-137.

Sewell, G.W.F. & Wilson, J.F. 1966, 'Verticillium wilt of the hop: the survival ofV. albo-atrum in soil', *Annals of Applied Biology,* vol. 58, no. 2, pp. 241-249.

Siddiqui, I.A. & Shaukat, S.S. 2002, 'Mixtures of plant disease suppressive bacteria enhance biological control of multiple tomato pathogens', *Biology and Fertility of Soils,* vol. 36, pp. 260-268.

Stockwell, V.O., Johnson, K.B., Sugar, D. & Loper, J.E. 2002, 'Antibiosis Contributes to Biological Control of Fire Blight by *Pantoea agglomerans* Strain Eh252 in Orchards', *Phytopathology,* vol. 92, no. 11, pp. 1202-1209.

Stover, R.H. 1950, 'The black rootrot disease of tobacco - Physiological specialization of *Thielaviopsis basicola* on *Nicotiana tabacum*', *Canadian Journal of Research,* vol. 28, pp. 726-738.

Stutz, E., Kahr, G. & Defago, G. 1989, 'Clays involved in suppression of tobacco black root rot by a strain of *Pseudomonas florescens*', *Soil Biology and Biochemistry,* vol. 20, no. 3, pp. 361-366.

Stutz, E.W., Defago, G. & Kern, H. 1985, 'Naturally occuring fluorescent *Pseudomonads* involved in suppression of black root rot of tobacco', *Disease Control and Pest Management,* vol. 76, pp. 181-185.

Sung, O.H. & New, P.B. 1998, 'Isolation of Azospirillum spp. from natural soils by immunomagnetic separation', *Soil Biology and Biochemistry,* vol. 30, no. 8-9, pp. 975-981.

Takakuwa, N., Yamane, K., Kinoshita, M., Oda, Y. & Ohnishi, M. 2003, 'Distribution of cerebroside in genus *Saccharomyces* and its closely related yeasts, and cloning of cerebroside metabolism-related genes', in *Advanced research on Plant Lipids*, N. Murata, M. Yamada, I. Nishida, H. Okuyama, J. Sekiya & W. Hajime (eds), Kluwer Academic Publishers, pp. 229-232.

Tanji, M., Namimatsu, K., Kinoshita, M., Motoshima, H., Oda, Y. & Ohnishi, M. 2004, 'Content and Chemical composition of Cerebrosides in Lactose-assimilating yeasts', *Bioscience, Biotechnology, Biochemistry,* vol. 68, no. 10, pp. 2205-2208.

Teixidó, N., Usall, J., Palou, L., Asensio, A., Nunes, C. & Viñas, I. 2001, 'Improving Control of Green and Blue Molds of Oranges by Combining Pantoea Agglomerans (CPA-2) and Sodium Bicarbonate', *European Journal of Plant Pathology,* vol. 107, no. 7, pp. 685-694.

Terras, F.R., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., VanLeuven, F., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. 1995, 'Small cysteine-rich antifungal proteins from radish: their role in host defense', *The Plant Cell,* vol. 7, pp. 573-588.

Terras, F.R.G., Schoofs, H.M.E., De Bolle, M.F.C., Van Leuven, F., Rees, S.B., Vanderleyden, J., Cammue, B.P.A. & Broekaert, W.F. 1992, 'Analysis of Two Novel Classes of Plant Antifungal Proteins from Radish (*Raphanus sativus* L.) Seeds', *Journal of Biological Chemistry,* vol. 267, no. 22, pp. 15301-15309.

Thevissen, K., Terras, F.R.G. & Broekaert, W.F. 1999, 'Permeabilization of Fungal Membranes by Plant Defensins Inhibits Fungal Growth', *Applied Environmental Microbiology,* vol. 65, no. 12, pp. 5451-5458.

Thevissen, K., Warnecke, D.C., Francois, I.E.J.A., Leipelt, M., Heinz, E., Ott, C., Zahringer, U., Thomma, B.P.H.J., Ferket, K.K.A. & Cammue, B.P.A. 2004, 'Defensins from Insects and Plants Interact with Fungal Glucosylceramides', *J. Biol. Chem.,* vol. 279, no. 6, pp. 3900-3905.

Tien, T.M., Gaskins, M.H. & Hubbell, D.H. 1979, 'Plant Growth Substances Produced by Azospirillum brasilense and Their Effect on the Growth of Pearl Millet (Pennisetum americanum L.)', *Applied and Environmental Microbiology,* vol. 37, no. 5, pp. 1016-1024.

Tsao, P.H. & Bricker, J.L. 1966, 'Chalmydospores of *Thielaviopsis basicola* as surviving propagules in natural soils', *Phytopathology,* vol. 56, pp. 1012-1014.

Van Loon, L.C. 1997, 'Induced resistance in plants and the role of pathogenesis-related proteins', *European Journal of Plant Pathology,* vol. 103, no. 9, pp. 753-765.

Van Loon, L.C. 2000, 'Systemic induced resistance', in *Mechanisms of Resistance to Plant Diseases*, A.J. Slusarenko, R.S.S. Fraser & L.C. Van Loon (eds), Kluwer Academic Publishers, Dordrecht

Vierheilig, H., Alt-Hug, M., Engel-Streitwolf, R., Mäder, P. & Wiemken, A. 1998, 'Studies on the attractional effect of root exudates on hyphal growth of an arbuscular mycorrhizal fungus in a soil compartment-membrane system', *Plant and Soil,* vol. 203, no. 1, pp. 137-144.

Vilas Alves, A., De Samblanx, G., Terras, F., Cammue, B. & Broekaert, W. 1994, 'Expression of functional *Raphanus sativus* antifungal protein in yeast', *FEBS Letters,* vol. 348, pp. 228-232.

Wang, H. & Davis, R.M. 1997, 'Susceptibility of Selected Cotton Cultivars to Seedling Disease Pathogens and Benefits of Chemical Seed Treatments ', *Plant Disease,* vol. 81, no. 9, pp. 1085-1088.

Ward, W.T., McTainsh, G., McGarry, D. & Smith, K.J. 1999, *The soils of the Agricultural Research Station at 'Myall Vale', near Narrabri, NSW with data analysis by fuzzy k-means*. Retrieved Technical report 21/99 from http://www.clw.csiro.au/publications/technical99/tr21-99.pdf

Whipps, J.M. 1997, 'Biological Control of Soil-borne Plant Pathogens', in *Advances in Botanical Research*, Academic Press, Birmingham, 26, pp. 1-134.

Whipps, J.M. 2001, 'Microbial interactions and biocontrol in the rhizosphere', *Journal of experimental botany,* vol. 52, pp. 487-511.

Wilkinson, C.A., Rufty, R.C. & Shew, H.D. 1991, 'Inheritance of partial resistance to black root rot in burley tobacco', *Plant Disease,* vol. 75, no. 9, pp. 889-892.

Yarwood, C.E. 1946, 'Isolation of *Thielaviopsis basicola* from soil by means of carrot discs', *Mycologia,* vol. 38, pp. 346-348.

Yarwood, C.E. 1981, 'The occurrence of *Chalara elegans*', *Mycologia,* vol. 75, pp. 524-530.

7 Appendix 1

Sequence of oligonucleotides used in the assembly PCR reactions (section 5.3):

Forward strand oligonucleotides

```
F1   5' cggt|ctcgag|aaaaga|atggctaagtttgcttctatcattgtccttctcttcgttgctcttgtcgtttttgct 3'
          |.XhoI.|.KEX..|Start

F2   5' gctttcgaaccaacaatggtggaagcacagaagttgtgtcagaggccaagtgggacatggtcag 3'

F3   5' gagtctgtggaaataataacgcatgcaagaatcagtgcattcgacttgagaaagcacgacatgggtcttgcaa 3'

F4   5' ctatgtcttcccagctcacaagtgtatctgttatttcccttgttaaagatctcggc 3'
```

Reverse strand oligonucliotides

```
R1   5’ gccg|agatct|ttaacaa|gggaaataacagatacacttgtgagctgggaagacatagttgcaagacccatgtcgt 3’
            |BglII |   Stop|

R2   5’ gctttctcaagtcgaatgcactgattcttgcatgcgttattatttccacagactcctgaccatgtcccacttg 3’

R3   5’ gcctctgacacaacttctgtgcttccaccattgttggttcttcgaaagcagcaaaaacgacaagagaa 3’

R4   5’ acgaagagaaggacaatgatagaagcaaacttagccattcttttctcgagaccg
```