Chapter 1 . Literature Review

1.1. General Introduction

T. basicola, a filamentous fungus, is a widespread, soil-borne plant pathogen. It has been reported to attack more than 200 ornamental and agricultural plants causing the disease, black root rot (Otani, 1962). In many hosts, the pathogen invades the epidermis and root cortex reducing the plant's capability to absorb nutrients (King and Presley, 1942). Although the pathogen was first detected in Australia on tobacco in 1942 (Simmonds, 1966), it has spread on a number of field crops in Australia via movement of spores attached to foot-wear or machinery wheels (Honess, 1994). The most serious and widespread epidemic of black root rot in Australia is cotton black root rot, which developed in New South Wales during the 1990s (Nehl *et al.*, 2004). It was estimated in 2004 that 97% of the cotton farms regularly surveyed in northern NSW have been infested by the pathogen and that by the year 2011, 95% of the cotton fields will be infested (Nehl *et al.*, 2004). Although this disease does not kill seedlings directly, yield losses up to 50% have been recorded in severely affected crops (Nehl, 2002). The incidence of black root rot could have an enormous impact on the Australian economy as Australia is the world's third largest cotton exporter (Dowling, 2003).

Past research on *T. basicola* has concentrated on finding better methods for controlling the root rot disease it produces on cultivated crops. Control strategies based on cultural practices, biocontrol agents, chemical fungicides, and genetically determined host resistance have not yet solved the loss of yield. As a soil-borne root pathogen, it has remained as an unsolved problem in agricultural systems in Australia and worldwide.

While the steps in the infection of host plants by *T. basicola* have been well documented (Hood and Shew, 1997c, b; Mims *et al.*, 2000), little is known about the genes involved in

plant-pathogen interactions. Moreover, no molecular biology tools have been developed for studying the infection process of *T. basicola.* The application of molecular genetic analysis to investigate mechanisms of the infection process will lead to the identification of resistance mechanisms that can be used to aid resistance breeding in host plants.

The focus of this review is to provide some background information on the host range and specificity of *T. basicola*, factors that promote and suppress black root rot, the steps that are involved in the infection process, the symptoms of black root rot and the current management procedures available for this disease. Understanding this area will allow the appreciation of *T. basicola*, the causal organism of black root rot, as a model organism for soil fungi because of its capability to interact with a wide variety of plants and its significant impact in the agricultural field. This review also highlights some of the pathogenicity genes that have been characterised during different stages of the infection process by filamentous fungi.

1.2. *T. basicola*

1.2.1. Characterisation of fungi

Fungi are eukaryotic microorganisms. They are heterotrophic (not autotrophic) meaning that they are unable to manufacture their own food by photosynthesis (nonphotosynthetic). Unlike animals (also heterotrophic), which ingest then digest, fungi digest by producing exoenzymes, then feed by absorbing simple nutrients small enough to pass through the fungal cell wall and across the plasma membrane (Hardham and Mitchell, 1998). Some fungi are unicellular, such as yeast, and others are multicellular known as filamentous fungi or moulds (Atlas, 1995). The filamentous fungi consist of a mass of filaments or vegetative cells that connect to each other resulting in multicellular branching tubular filaments known as hypha. Integrated masses of hyphae are called mycelia. Hyphae

contain individual compartments formed by crosswalls called septa, but in some fungi, hyphae are coenocytic (aseptate) (Atlas, 1995). The hyphae are surrounded by cell walls composed of chitin, 1,3-β-glucan and 1,6-β-glucan, mannan and proteins. However, cell wall composition varies markedly between species of fungi. The fungal cell wall is a highly dynamic structure subject to constant change, for example, during cell expansion and division in yeasts and during spore germination, hyphal branching and septum formation in filamentous fungi (Adams, 2004). Fungi propagate by spores as a result of asexual and sexual reproduction (Atlas, 1995). Many of the fungal pathogens of plants (and humans) belong to the group of filamentous fungi that exhibit dimorphism (capable of reversible transitions between yeast and hyphal forms) (Gow *et al.*, 2002).

Filamentous fungi use polar growth. After a period of isotropic or symmetric expansion, filamentous fungi switch permanently to apical extension and a germ tube emerges. New material is added exclusively to the apex, resulting in an extending tubular cell, or hypha. Branches also emerge from the main hypha, establishing additional axes of polarity (Momany, 2002).

T. basicola is a filamentous soil-borne fungus that attacks plants causing the soil-borne root disease black root rot (Shew and Meyer, 1992). Soil-borne fungi are fungi that are detected in soil and roots of plants.

Plants are the primary producers of the ecosystem and thus are the major source of food to all animals as well as many microorganisms. Losses in crop production due to plant diseases average 13% worldwide and severely limit production of food. The 11,000 different diseases documented in plants, are caused by 120 genera of fungi, 30 types of viruses and eight genera of bacteria (Montesinos *et al.*, 2002).

1.2.2. Host range

T. basicola is a very common fungus. Plant health authorities in over 46 countries have reported it as a pathogen infecting 137 plant species in 33 plant families (Otani, 1962). It is found in undisturbed habitats as well as in crops and can be commonly found in both uncultivated and cultivated soils (Yarwood, 1974, 1981). The fungus is commonly found on plants from the Fabaceae, Solanaceae, Malvaceae and Curcubitiaceae families (Shew and Meyer, 1992). Important agricultural hosts include cotton, tobacco, beans, carrots, soybeans, peanuts and lettuce. *T. basicola* also infects ornamental plant species which include sweet peas and pansies. Although the fungus is a facultative parasite, it can also associate with hosts in a non-pathogenic manner (Yarwood, 1974).

1.2.3. Host specificity

Host-pathogen specificity as defined by Zhou and Hyde (2002) is a relationship in which a fungus derives its nutrition from a live host plant during some phase of its life cycle, and is restricted to a particular host or a group of related species, but does not occur on other unrelated plants in the same habitat. Many fungal pathogens are host-specific and this view has been supported by many reviews (Wolpert *et al.*, 2002; Zhou and Hyde, 2002).

Host-pathogen specificity has also been reported to exhibit different degrees of genetic specificity (Kirchner and Roy, 2002). For example, some soil-borne plant pathogens exhibit low specificity, infecting a wide range of host genotypes (Kirchner and Roy, 2002). The molecular mechanisms that are involved in fungal host range specificity are not fully understood. However, a number of virulence determinants have been identified to be host specific. For example, some isolates of the ascomycete, *Nectria haematococca* contain pisatin demethylase and pea pathogenicity (*PEP*) genes that are found on dispensable pisatin demthylase chromosomes that encode the ability to detoxify phytoalexins of pea

whereas, other strains encode other genes present on non-pisatin demethylase-containing chromosomes that cause pathogenicity on other hosts such as ripe tomato and mature carrots (Funnel and VanEtten, 2002).

Evidence of host specificity in *T. basicola* isolates has been reported to be related to differences in pathogenicity (Keller and Shanks, 1955; Lloyd and Lockwood, 1963; Tabachnik *et al.*, 1979; O'Brien and Davis, 1994; Bai *et al.*, 1996). Keller and Shanks (1955) found that *T. basicola* strains that attacked tobacco did not attack poinsettia and *vice versa*. Lloyd and Lockwood (1963) reported that *T. basicola* strains from orange, pea and poinsettia were moderately to highly pathogenic on bean and pea but nonpathogenic on tobacco plants. In contrast, strains from tobacco were highly pathogenic on tobacco but nonpathogenic on bean plants. One tobacco strain was mildly pathogenic on pea plants. Tabachnik *et al.* (1979) reported that although host specificity was found for some isolates of *T. basicola*, isolates obtained from various hosts were not necessarily specific for these hosts. The specificity they observed could be due to differences in stimulation of spore germination, or differences in pathogenicity.

Differences observed in pathogenicity between different isolates are due to differences in the genotype between the isolates. Results from RAPD analysis conducted by Punja and Sun (1999) support the possibility of the occurrence of genetically distinct strains of the pathogen that may be more adapted to specific hosts.

Specific host preferences among isolates of *T. basciola* have previously been demonstrated experimentally. Specificity of hosts by *T. basicola* may occur at the plant species or at the cultivar level. At the species level, the tobacco species *Nicotiana debneyi* was shown to be completely resistant to a wide range of *T. basicola* strains while another tobacco species *N. tabacum* showed varying levels of susceptibility to the disease (Bai *et al.*, 1996). Cultivar specificity has been demonstrated on lettuces (*Lactuca sativa* L.) with the

cultivars Monaro and Centenary being resistant, while others like Classics and Yatesdale being highly susceptible to infection by *T. basicola* (O'Brien and Davis, 1994). In a more detailed and thorough study, Punja (2004) used 27 isolates of *T. basicola* to assess the degree of pathogenicity on detached bean leaves as host tissues. His results also demonstrated that there were pronounced differences in pathogenicity among isolates. These differences were correlated with differences in inoculum level, culture age, colony morphology, leaf age and bean cultivar. Cultivar resistance toward fungi is reported to be pathogen-specific and controlled by plant hosts carrying single dominant resistance (R) genes that can recognise pathogens carrying single dominant genes (avirulence genes) resulting in a "gene for gene interaction" (Heath, 1991). Pathogens that are recognised in this way and fail to cause disease are called 'avirulent pathogens' the host is called 'resistant' and the interaction is called 'incompatible'. In the absence of gene-for-gene-recognition, due to absence of the avirulence gene in the pathogen and/or of the R gene in the host, the pathogen is 'virulent', the host is 'susceptible', and the interaction is 'compatible' (Mysore and Ryu, 2004).

Identifying the avirulence factors in the pathogen could unravel molecular mechanisms of disease resistance in plants. In fungal plant pathogens, only a few avirulence factors have been identified. The majority of these are small cysteine-rich proteins from leaf-invading fungi such as *Cladosporium fulvum*, which is currently the most-developed model for fungus-plant "gene for gene interactions". Many resistance and avirulence genes have been the subject of intense investigation in recent years (Hammond-Kosack and Jones, 1997; Wolpert *et al.*, 2002; Glazebrook, 2005) . However, only a few avirulence factors have been found in root-invading fungi including such as *Fusarium*. Rep *et al.* (2004) recently identified a small cysteine-rich protein secreted by *F. oxysporum* during colonization of xylem vessels that is required for the I-3-gene mediated resistance in tomato.

1.2.4. Variability in culture

Specific host preferences among strains of *T. basicola* have prompted researchers to conduct morphological variation studies. These studies have been mainly conducted on colony morphology, chlamydospore size, septation and pigmentation (Punja and Sun, 1999). Variations were reported to occur either during growth of the fungus in pure culture or among isolates originated from infected host plants in nature. Morphological and molecular approaches have been used to assess the degree of variation in *T. basicola* (Punja and Sun, 1999).

1.2.4.1. Morphological variability

In culture, pronounced morphological variations have been observed when single chlamydospores of *T. basicola* were subcultured on different agar media (Stover, 1950b; Huang and Patrick, 1971). Interestingly, the resultant colonies differed in colony appearance, color, zonation, growth rate, production of spores, length of chains of chlamydospores and pathogenicity. More surprisingly, the production of different variants did not only occur in old cultures but also from adjacent cells within the same chlamydospore chain (Huang and Patrick 1971). Both mass-transfer cultures and cultures derived from single endoconidia have been documented to exhibit variability (Stover, 1950a).

Different colony types of *T. basicola* have been recognised according to their morphology. Stover (1956) found that the fungus exists in nature mainly as two distinct types; the grey and the brown biotypes. Each could give rise to the other during subculturing. Grey and brown colony types of this pathogen have also been described by Punja and Sun (1999) and each were seen to give rise to the other. The most common variants observed in these studies were the albino colonies that arose from a wild-type brown isolate. What triggers this variation both in nature and culture is still unknown.

Morphological variations of *T. basicola* have been attributed to the presence of double stranded RNA. Park *et al.* (2006) reported that there are three groups of viruses in *T. basicola* and that the transmission of dsRNA elements through conidia of *T. basicola* was highly efficient. Double stranded RNA has also been reported to occur in a number of different plant pathogenic fungi (Nuss and Koltin, 1990). In wild-type *T. basicola* strains, the presence of dsRNAs was found to significantly enhance sporulation, alter colony morphology and reduce growth rate (Bottacin *et al.*, 1994; Punja, 1995).

1.2.4.2. Molecular variability

The application of molecular methods to study variability has revealed a high level of genetic diversity within *T. basicola* isolates (Punja and Sun, 1999; Pattemore and Aitken, 2000). Using both methods, morphological variation and random amplified polymorphic DNA (RAPD) analysis, Punja and Sun (1999) were able to reveal that isolates derived from one host share greater genetic similarity than isolates from different hosts. Thus, the hypothesis put by Punja and Sun (1999) is that host specialization may lead to the differentiation of *T. basicola* isolates into intraspecific groups. The high degree of intraspecific variation observed among different strains of *T. basicola* was suggested to be the result of a high mutation rate and the presence of transposable elements. In Australia, a preliminary study conducted by Pattemore and Aitken (2000) used the RAPD technique to test the hypothesis that documented differences in host specificity of *T. basicola* isolates affecting cotton is partially explained by genetic variation within and among populations of the pathogen due to different evolutionary histories. RAPD profiles revealed that *T. basicola* isolates taken from two cotton-growing regions clustered into two distinct groups suggesting independent evolution of *T. basicola* in the regions they were isolated from (Warren, NSW and Goondiwindi, Qld). Isolates from a third cotton-growing region (Narrabri) did not cluster and were distributed between the other two regions (Warren, NSW and Goondiwindi, Qld), suggesting migration and gene flow between these regions.

Lettuce isolates also clustered into a distinct group. Their results demonstrated that populations of the pathogen formed distinct groups based on geographic origin and host. Therefore, there is evidence to suggest that the results of Pattermore and Aitken (2000) corroborate the hypothesis put forward by Punja and Sun (1999) and the recent findings of Geldenhuis *et al.* (2006). Geldenhuis *et al.* (2006) used co-dominant polymorphic markers developed for *T. basicola* to study the population diversity of *T. basicola* isolates in South Africa and to compare these isolates with isolates from other countries. They found that three different regions in South Africa were represented by a genetically uniform population of *T. basicola* isolates and that a relatively large collection of these isolates included only two different genotypes that were closely related and subdivided based on their host of origin. Isolates from the Netherlands originated from different hosts and represented a different genotype. Geldenhuis *et al.* (2006) suggested that there was some evidence for host specialization of the different genotypes.

1.3. Morphology of *T. basicola*

The spores of *T. basicola* have been defined by several studies using the light microscope as two distinct types of asexual spores present in artificial solid media and on diseased plants. These include: hyaline cylindrical liberated conidia and thick-walled pigmented chlamydospores (Punja, 1993). A conidium is a non-motile, asexual spore formed at the tip or side of a sporogenous cell (Alexopoulos and Mims, 1979). The conidia of *T. basicola* are also known as phialospores or endoconidia. They are known as phialospores because they are produced from phialides. A phialide produces conidia in a basipetal fashion from an open end. Basipetal fashion means that the oldest conidium is at the tip and the youngest at the base (Alexopoulos and Mims, 1979). They are referred to as endoconidia when the conidia exist as microconidia within the phialide. Liberated single endoconidia are variable in size and generally have slightly rounded ends and can be seen scattered in

large numbers. The walls of the endoconidia appear to be structurally distinct from the wall of the phialide. Not all phialides contain endoconidia, but can exist as empty cylinders (Punja, 1993).

The chlamydospores of *T. basicola*, sometimes referred to as macroconidia, aleuriospores or arthoconidia, are larger and darker in appearance than the liberated single endoconidia (Lindsey, 1981). A chlamydospore can be defined as a thick-walled thallic conidium that generally functions as a resting spore (Alexopoulos and Mims, 1979). Chlamydospores are composed of thick-walled compartments and produced in branched chains at the tip of the hyphae or can be seen attached at intercalary positions (Figure 1.1). The number and size of individual spores in the chains can vary according to the isolates (Punja and Sun, 1999).

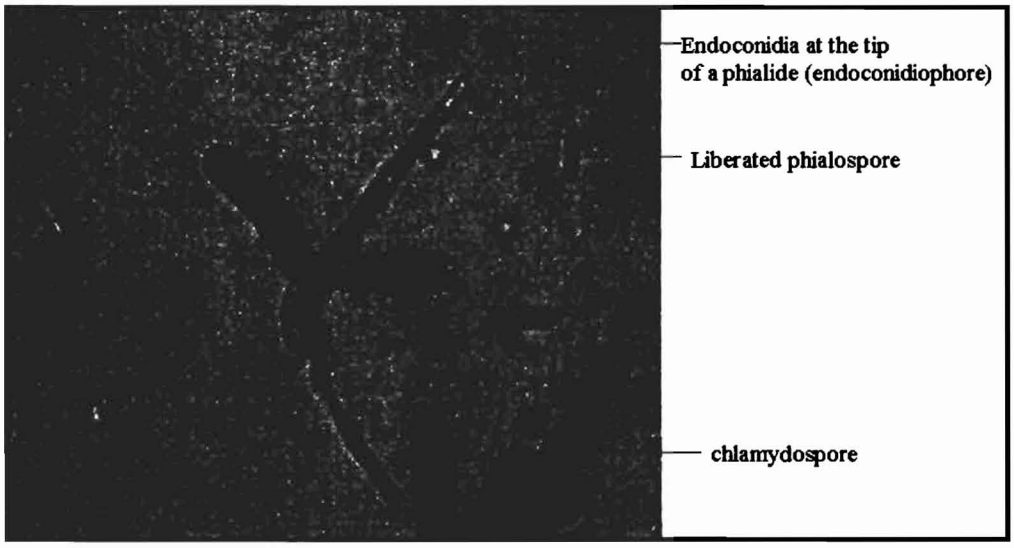

Figure 1.1. An endoconidium produced inside a phialide
Adapted from http://comp.uark.edu/~rothrock/thielaviopsisbasicola.html

Riggs and Mims (2000) have presented ultrastructual details of chlamydospore development from root hairs of infected pansies. Their findings revealed that the development of a chlamydospore begins with a lateral bud or germ tube that ruptures the outer wall layer of the parent hypha giving rise to a sporogenous cell. The sporogenous cell is covered by a wall that is continuous with the inner layer of the parent hypha wall. A

nucleus then enters the sporogenous cell and a septum is formed to delimit it from its parent hypha. The sporogenous cell then slightly elongates and its nucleus undergoes mitosis creating two uninucleate cells. A transverse septum then forms to divide the original cell into an apical cell and a basal cell. The basal cell ceases to develop while the apical cell continues to elongate and its nucleus again divides by mitosis to produce two nuclei. Further septation occurs to divide the apical cell into two cells and this process is continued until a chain of cells is formed. The basal cell can also give rise to a bud which can grow into another sporogenous cell (Riggs and Mims, 2000).

The thick walls of the chlamydospores are thought to enable the spores to survive periods of adverse environmental conditions such as high and low temperatures, lack of moisture and exhaustion of food supply which also prevent or delay germination (Atlas, 1995). Long-term survival of *T. basicola* in soil occurs mainly through the production of the melanized, thick-walled, robust chlamydospores. The small, less robust, endoconidia do not survive for long periods in soil as the hyaline, thin-walled endoconidia are more subject to lysis by microbial action than chlamydospores. However, a small percentage of endoconidia may remain dormant and viable as long as chlamydospores (Chittaranjan and Punja, 1994).

1.4. The genus *Thielaviopsis*

Nag Raj and Kendrick (1975) reported that *T. basicola* endoconidia produced from phialides have all the essential characteristics of a genus termed the *Chalara* complex of the Hyphomycetes. The *Chalara* complex represents fungi which have a phialospore state (endoconidial) and differ in presence or nature of the aleuriospore state (chlamydospores). Therefore taxonomically, they placed *T. basicola* in the genus *Chalara* with a new species name, *Chalara elegans.*

More recently, Paulin-Mahady and Harrington (2000) used rDNA sequences to study *Chalara* species. They discovered that most of the *Chalara* species without known teleomorphs have been found to belong within the Leotiales and only a few belong to the teleomorph genus *Ceratocystis*. Therefore, they suggested that the genus *Chalara* is not an appropriate name for anamorphic species related to *Ceratocystis*. In 2002, Paulin-Mahady *et al.* (2002) placed four anamorphic *Chalara* species including *C. elegans*, as a monophyletic grouping within the teleomorph genus *Ceratocystis* (perithecial ascomycete) and within the anamorph genus *Thielaviopsis* because these four species form aleurioconidia typical of the anamorph genus *Thielaviopsis*. The phylogenetic and taxonomic evaluation of *Chalara* conducted by Paulin-Mahady *et al.* (2002) confirms *Thielaviopsis basicola* as the correct name and this name will be used in this thesis. However, one must alert readers that *Chalara elegans* has been used interchangeably for *T. basicola* by some researchers. The genus *Thielaviopsis* amended by Paulin Mahady *et al.* (2002) includes the presence or absence of chlamydospores, which when present may be produced in chains (*Thielaviopsis*) or singly (as *Chalaropsis).* To date, only four *Thielaviopsis* species, *T. thielavioides*, *T. populi*, *T. ovoidea* (all produce chlamydospores singly) and *T. basicola* (produces chlamydospores in chains) have been identified. They are soil-borne, root pathogens that do not depend on insects for dispersal. The feature chlamydospores are thought to be associated with species that are soil-borne (Paulin-Mahady *et al.*, 2002).

1.5. Isolation of *T. basicola*

Yarwood (1946) designed a technique of isolating *T. basicola* from soil, which since then has prompted other researchers to improve the method. His method utilised freshly cut carrot root disks incubated on the soil surface. Within 72-96 h, colonization of the tissue occurred and the pathogen is easily recovered. Although this technique is very sensitive, it

provides a qualitative but not quantitative assessment of pathogen population in soil. Another major disadvantage is that it supports growth of other fungi (Lloyd and Lockwood, 1962). Alternatively, several semi-selective agar media have been described for enumeration of populations of *T. basicola* in soil. Isolation of *T. basicola* from soil as well as host tissue has been reviewed by Shew and Meyer (1992).

1.6. Geographical distribution of *T. basicola*

T. basicola was first described in Britain in 1850 and since then it has been reported in all continents, mainly in America, Canada, Europe and Australia. However, in Australia it was not reported from natural habitats. In Australia, *T. basicola* appears to be widespread in agricultural soils in all states except the Northern Territory (Honess, 1994). This fungus was first recorded in 1930 in the state of Queensland as a pathogen of sweet pea (Simmonds, 1966) and since then it has been recorded also in tobacco, *Lupinus angustifolias* L and lettuce (O'Brien and Davis, 1994). *T. basicola* is also widespread in all cotton growing areas of New South Wales and on the Darling Downs of Queensland (Allen and Lonergan, 1997). It is believed that the infection of some crops in Australia, especially lettuce, originated from infected peat imported from Canada (Graham and Timmer, 1991), New Zealand, Ireland and other Northern European countries (Pattemore and Aitken, 2000). The significant impact of *T. basicola* in causing black root rot on a variety of plants has led to extensive study of this organism, and its mode of pathogenesis and ecology are emerging.

1.7. Symptoms of black root rot

T. basicola can be easily detected using light microscopy. The symptoms of the disease depend on a wide range of factors: the susceptibility of the host species and cultivar, the strain of *T. basicola* and the inoculum levels present at the time of the infection. *T.*

basicola is essentially an invader of below ground plant parts, however, above ground symptoms are also visible.

1.7.1. Below ground symptoms

King and Presley (1942) wrote a substantial analysis of below ground disease symptoms of cotton seedlings as well as mature cotton plants. Roots of affected cotton seedlings display a purplish-black discolouration of the affected tissues. Root seedlings displaying mild symptoms reveal that the endodermis is capable of preventing passage of the invading fungus into the stele (Garber *et al.*, 1985). In seedlings with severe symptoms, the stele of the main root is discolored and portions of it disintegrate, forming cavities. As the disease becomes more severe, the cortex of the root becomes black and appears in a shrunken condition. The entire root is then so fragile that it can easily be pulled from the ground (King and Presley 1942). Black root rot does not cause seedling and plant death. However, severe black root rot may increase the plant's susceptibility to other fungal pathogens e.g. *Rhizoctonia* and *Pythium*, which can kill the plant (Rourke and Nehl, 2001).

In mature cotton plants, the first sign of the disease is an abnormal swelling of the main root. Though the bark tissue on the taproot appears healthy, the stele or the tissues within appear to be rotting, revealing a brown to purplish-black colour (King and Presley, 1942). This swelling and discolouration is due to the production of chlamydospores in the stele (Figure 1.2). The damage reduces the plant's capacity to absorb nutrients. Evidence of external decomposition can be seen on the lateral roots. A general decay then occurs, as the plant is more susceptible to attack by other pathogens (Mauk and Hine, 1988).

On soybean seedlings, black discolouration occurs on the upper taproot and on lateral roots. Examination of the roots of soybeans seedlings under a dissecting microscope revealed the presence of many chains of dark chlamydospores. The symptoms did not

progress beyond the upper region of the taproot, close to the junction with the stem (Mondal *et al.*, 2004).

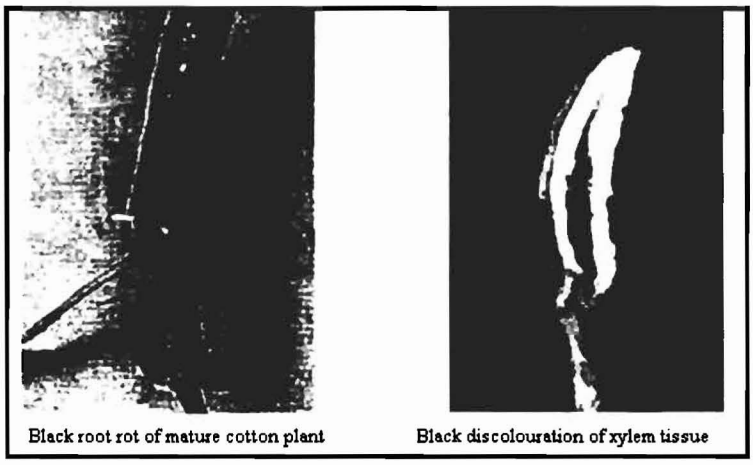

Figure 1.2. Black root rot in a mature cotton plant.

(Photo by David Nehl)

http://www.rbgsyd.nsw.gov.au/science/hot_science_topics/Soilborne_plant_diseases/Vietnam_template3/Thielaviopsis_Diseases

1.7.2. Above ground symptoms

The above ground symptoms of cotton black root rot are stunted and unhealthy seedlings. The leaves are often small and cupped, pale green, purplish along the veins and show marginal browning and burning. Gradually, the lower leaves wither and drop off (King and Presley, 1942). Usually, the above ground symptoms begin with a reduction in growth and increasingly apparent foliage discolouration and wilting. Swelling of the stem directly above the soil surface also occurs (King and Presley, 1942). This pathogen has also been described as a pathogen on leaves of tobacco (*Nicotiana tabacum* L.) that causes distinct necrotic lesions that resemble local lesions caused by viral pathogens such as the *Tobacco necrosis virus* (TNV) (Hecht and Bateman, 1964). These lesions also closely resemble lesions observed in a more recent study by Punja (2004) on bean leaves, confirming that foliar tissues are also susceptible to this pathogen.

Unlike *T. basicola,* which is essentially an invader of roots, *Magnaporthe grisea*, an ascomycete fungus and the most destructive pathogen of rice, is essentially an invader of leaf and is not regarded as an economically important root pathogen. It has been reported to infect many agriculturally important cereals such as wheat, rye and barley, causing diseases called blast disease or blight disease. However, it has also recently been demonstrated that in addition to infecting plants through the leaf, *M. grisea* can also infect plant roots (Dufresne and Osbourn, 2001; Sesma and Osbourn, 2004). In roots, the blast fungus invades the plant's vascular system, growing inside the xylem and phloem and blocking the transport of nutrients and water from the roots. Infection of root and vascular tissues has the potential to kill the plant by cutting off the supply of water and nutrients to the root (Sesma and Osbourn, 2004).

T. basicola and *M. grisea* provide interesting models for further studies to determine if any correlation exists between leaf and root necrosis. Such findings could lead to the application of leaf inoculations that may be useful for preliminary screening of plants resistant to fungal disease.

1.8. Factors promoting and suppressing black root rot

Factors that contribute to disease severity and suppression mostly include soil properties. Many researchers have investigated the chemical, physical and biological properties of soil. The relationship between various abiotic and biotic soil factors and the capacity of the soil to allow the survival of soil-borne pathogens and the occurrence of disease is known as soil receptivity. Both abiotic and biotic soil factors can be either conducive or suppressive to a certain pathogen.

1.8.1. Abiotic factors

Abiotic soil factors include soil pH intercorrelated with available calcium, aluminium, phosphorous and ammonium levels in the soil. Soil pH, soil temperature and soil type have also been attributed to abiotic factors (Colhoun, 1973).

1.8.1.1. Soil pH, calcium, aluminium and phosphates

Severe black root rot associated with soil pH that is greater than 5.6 is well documented for tobacco and other host species (Bateman, 1962). Soil pH is known to indirectly affect the distribution and activity of soil microorganisms, including plant pathogens by altering the solubility of ions in the soil (Kaufmann and Williams, 1964). The relationship between black root rot severity and soil pH is that as the soil pH increases, the black root rot also increases. Soils with a pH value above 5.6 promote the disease whereas soils with pH values of 5.2 or lower suppress the disease. Alkaline soils containing high levels of calcium and nitrates enhance *T. basicola* pathogenicity (Oyarzun *et al.*, 1998).

Acidic soils that suppress the disease are seen to contain high levels of exchangeable aluminium, carbon, soluble magnesium and phosphates. Acidic soils with high levels of exchangeable aluminium have been shown to inhibit hyphal growth and spore germination (Meyer *et al.*, 1994). However, *T. basicola* shows maximal growth rate at a pH just below 5.0 *in vitro* on artificial media (Bateman, 1962) and acidic soils that contain high levels of calcium and low levels of aluminium have been reported to positively influence the development of black root rot (Meyer and Shew, 1991). Therefore, this indicates that the pH is not the only factor that suppresses or promotes black root rot in acidic soils. The abiotic characteristics of soil can operate in the suppression of plant diseases directly or indirectly through the impact on soil microbial activity (Mazzola 2002). It is thought that rhizosphere microorganisms in plants growing in acidic soils may be more inhibitory to the

growth of *T. basicola* than microorganisms thriving in alkaline or neutral soils (Papavizas, 1968).

1.8.1.2. Soil temperature

The influence of soil temperature on natural populations of *T. basicola* has been examined. A number of previous studies have shown that cool, soil temperatures, less than approximately 26°C, increase disease severity for cotton (Blank *et al.*, 1953; Mauk and Hine, 1988), tobacco (Johnson and Hartman, 1919) and poinsettia (Bateman and Dimock, 1959). The results of these studies and others have been interpreted by Lloyd and Lockwood (1963). Their investigation of the relationship of soil temperature and development of the disease indicated that temperatures unfavourable for growth of the host appeared to be favourable for development of black root rot. They suggested that the optimum temperature for fungal growth was not a factor in pathogenesis and that *T. basicola* was most destructive when plants were grown under unfavourable conditions e.g. tobacco grown at too low temperatures. Such adverse conditions resulted in lower resistance of the plant to infection.

1.8.1.3. Soil type

Soil type has also important implications for soil-borne diseases. It was previously documented that *T. basicola* infected crops grown in clay rather than in sandy soils (Papavizas and Adams, 1969). This is because clay soils retain moisture for longer periods supporting the germination of chlamydospores. Black root rot is more severe for cotton in wet, poorly drained, soils than well-drained soils (King and Presley, 1942). Fine textured-soils have been reported to increase black root rot severity on soybean (Lockwood *et al.*, 1970) compared with disease severity in coarser textured soils. Loams have been reported to have lower rhizosphere populations of the pathogen than sandy loams (Papavizas and Davey, 1961). However, recent studies questioned the ability of soil texture to influence

pathogen survival or disease development. Soil texture treatments conducted by Rothrock (1992) included fumigated (98% methyl bromide + 2% chloropicrin) sand or soil to nonfumigated soil in different ratios. His results demonstrated that soil texture did not play a significant role in the survival of *T. basicola* populations or the development of black root rot on cotton. He therefore concluded that the relationship of black root rot with soil texture may be connected to other factors, such as soil compaction, water infiltration rates and soil aeration.

1.8.2. Biotic factors

Biotic factors include microorganisms thriving in soil and organic amendments in soil. Soils have been amended to either promote or suppress disease. Suppressive soils are defined as soils in which a pathogen is present and can survive but does not cause disease on a susceptible host (Mazzola, 2002). For example, heavy soils, with a higher content of ash and silt have been reported to be more conducive to *T. basicola* (Oyarzun *et al.*, 1998). However, there have been contradicting reports on soil amendments using organic matter. Whereas some studies show that introducing high organic matter content into soil increases the persistence of *T. basicola*, other studies did not find that to be the case (Papavizas, 1968). The introduction of organic amendments, especially alfalfa hay and corn stover, to soil had been very effective in the decline of inoculum density of the pathogen and therefore in reducing the disease severity of black root rot (Papavizas, 1968).

Research has also looked into natural soil in the environment without amendments that suppress disease. For example, natural soil in the Morens region of Switzerland has been found to be naturally suppressive to black root rot of tobacco. The suppressive agents, fluorescent *Pseudomonas* bacteria, are found to occur in the rhizosphere (Stutz *et al.*, 1986; Stutz *et al.*, 1989). In a similar and recent study, a higher number of fluorescent pseudomonads in the rhizoplane of peas has been associated with suppressiveness to *T.*

basicola (Oyarzun *et al.*, 1998). *Pseudomonas* species are known to synthesize a wide variety of antibiotic compounds that suppress root diseases e.g. phenazine-1-carboxylic acid, diacetylphlorogucinol (Phl), hydrogen cyanide (HCN), oomycin, pyoluteorin and pyrrolnitrin (Laville *et al.*, 1992). CHA0, a mutant strain of *P. fluoresens* which lacks the gene *gacA* (for global antibiotic and cyanide control) has been used to study the suppression of diseases caused by *F. oxysporum, Rhizoctonia solani, Pythium ultimum, Aphanomyces euteiches* and *T. basicola* (Defago *et al.*, 1990; Defago and Keel, 1995). This mutation is known to pleiotropically block the production of Phl, HCN and pyoluteorin, reducing the suppression of black root rot of tobacco in gnotobiotic chambers (Laville *et al.*, 1992). Phl and HCN have been shown to play a key role in the protection of tobacco against *T. basicola* in a gnotobiotic system, mimicking Morens soils and where sufficient iron is present in vermiculitic clay minerals (Keel *et al.*, 1989; Stutz *et al.*, 1989). Fluorescent pseudomonads producing antifungal Phl and /or HCN are also present in natural soils conducive to tobacco black root rot. However, the population size of Phl^+ isolates in the rhizosphere and roots of tobacco plants grown in conducive soils is reported to be lower than in suppressive soils (Ramette *et al.*, 2003).

Studies suggest that some species of arbuscular mycorrhizal fungus reduce the incidence of subsequent infection by *T. basicola* and that this effect is dependent on the cultivar or variety of the host plant involved (Davis, 1980; Giovanetti *et al.*, 1991).

However, not all natural soils that contain microorganisms suppress disease. For example, another biotic factor that was found to influence the ability of *T. basicola* to cause disease is the root-knot nematode *Meloidogyne incognita*. This nematode has been reported to cause serious damage to cotton plants in studies conducted in America (Walker *et al.*, 1998, 1999, 2000; Wheeler *et al.*, 2000). When both the nematode and the fungus appear together, they cause more damage to cotton plants than either pathogen alone. It is thought

that the nematode increases the access of *T. basicola* to plant vascular tissue (Walker *et al.*, 1998, 1999, 2000). The presence of this nematode has not been reported for black root rot of cotton in Australia.

1.9. Fungal pathogenicity in plants

Plant-fungus interactions are generally described as susceptible if the fungus produces characteristic symptoms and reproduces on/within the plant, or resistant, if the plant can respond to the infection by restricting symptom development and pathogen reproduction. Plant parasitism is a direct result of competition for nutrients and niche (Dean, 1997). Many studies have reported that pathogenic fungi show extreme diversity: They belong to many different families each with many different genera (Schafer, 1994; Dean, 1997; Idnurm and Howlett, 2001; Tucker and Talbot, 2001). They also show different modes of infection and pathogenesis as well as have different nutritional requirements (Schafer, 1994; Dean, 1997). They colonize different parts of the plant, above or below ground. However, recent studies describe some fungal pathogens including *M. grisea* and *T. basicola*, as pathogenic on leaves as well as on roots. Most of the literature on *T. basicola* reports this fungus as an invader of epidermal and cortical tissues of roots of plant species and therefore research has concentrated and provided a considerable amount of detailed description on the infection process of *T. basicola* on host roots. Only a few studies have described this fungus as a foliar pathogen. The observation of Punja (2004) on the colonization of *T. basicola* on bean leaves were mainly based on the host tissue response to infection rather than the infection process itself. Therefore, the following section will provide more emphasis on the infection process of *T. basicola* on roots. However, the colonization of different parts of the plant above the ground by other pathogenic fungi has been extensively reviewed (Schafer, 1994; Dean, 1997; Tucker and Talbot, 2001).

1.9.1. Infection process by *T. basicola* on roots

Some progress has been made in elucidating the life cycle (summarized in Figure 1.3) and steps of penetration and infection of host plant roots by *T. basicola* (Figure 1.4). These steps involve the following; germination of *T. basicola* spores, growth of *T. basicola* towards the host plant roots, surface contact of *T. basicola* to host roots, penetration into host root hairs or epidermal cells, establishment of biotrophic phase and conversion to necrotrophy.

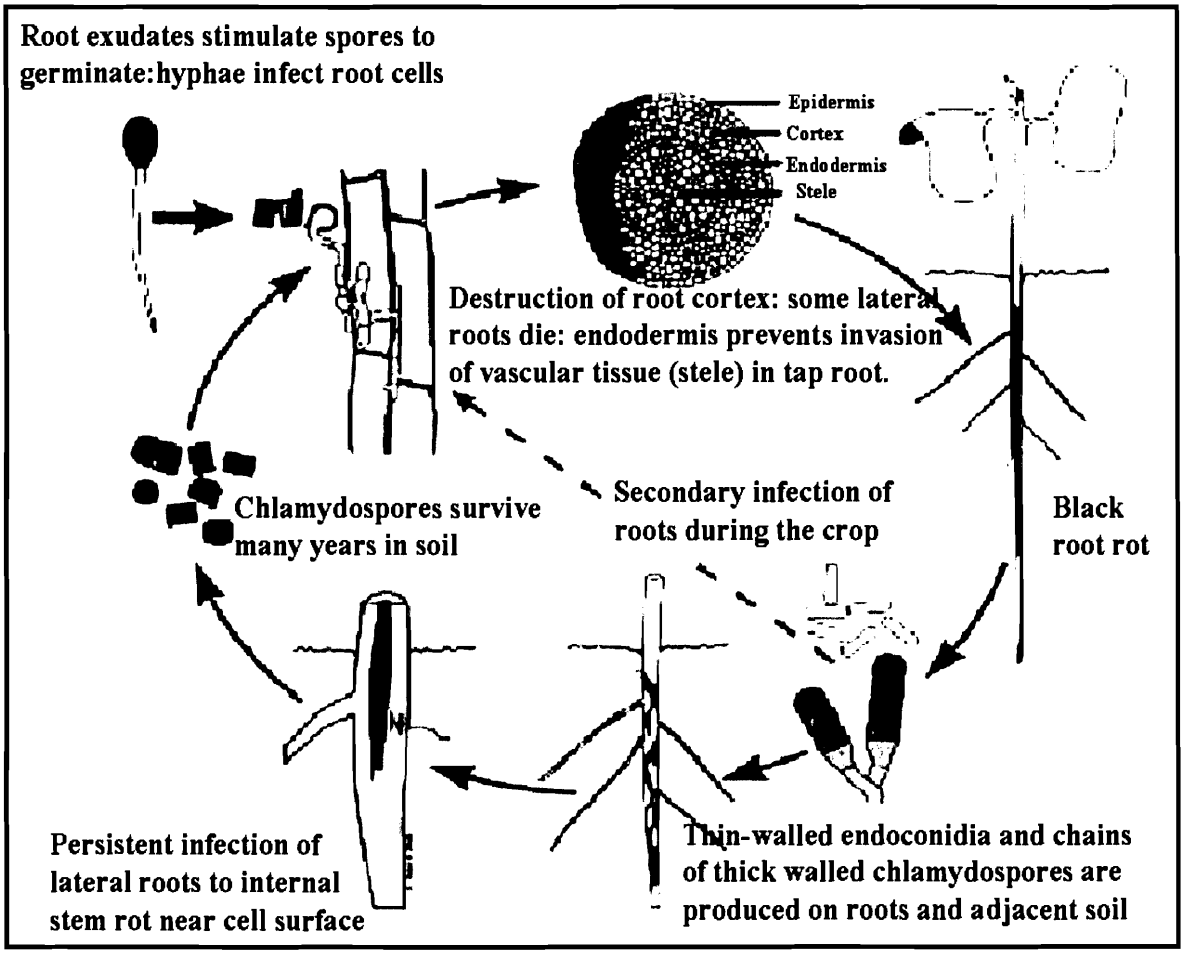

Figure 1.3. Schematic illustration of the life cycle of *Thielaviopsis basicola* leading to black root rot

Adapted from Nehl *et al.* (2000).

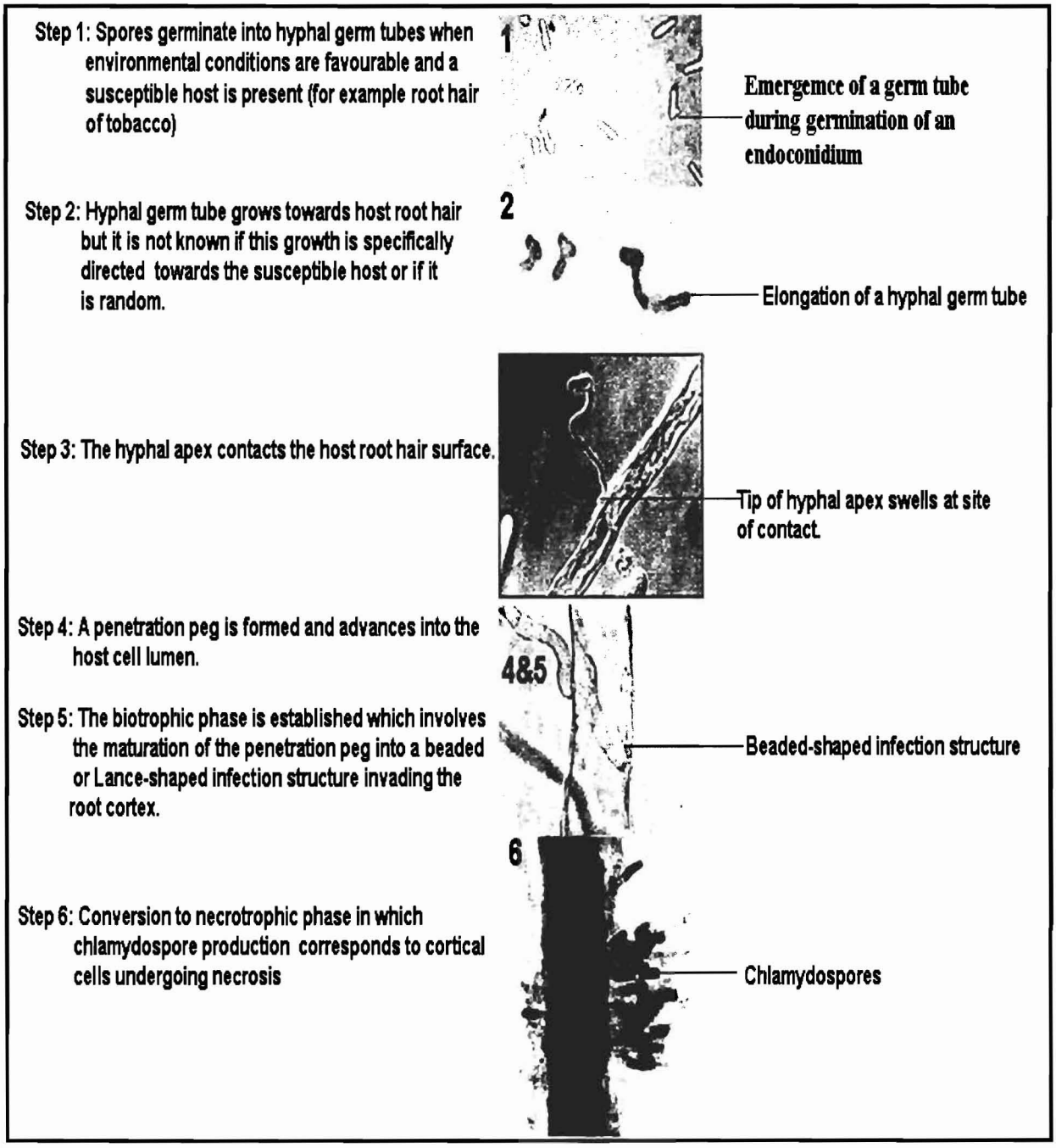

Figure 1.4. Infection process of *Thielaviopsis basicola* leading to black root rot

Steps 3-5 adapted from (Hood and Shew, 1997b). Step 6 adapted from http://www.cals.ncsu.edu/course/pp728/Thielaviopsis/symptoms_of_black_root_rot.html

1.9.1.1. Germination of *T. basicola* spores

Jennings and Lysek (1999) consider germination of fungal spores as the last and equally crucial step in the process of reproduction. On the other hand, one may consider germination as the first step of the life cycle in *T. basicola.* In solid or broth media endoconidia are produced within 24 h, whereas chlamydospores are not produced until after 3 days (Shew and Meyer, 1992). Previous reports have described that endoconidia of *T. basicola* germinate to produce only mycelia. However, Punja (1993) observed that the process of phialospore germination in *T. basicola* has two distinct modes based on the presence or absence of exogenous nutrients. In the presence of nutrients, the released endoconidia produce germ tubes that branch and develop into extensive hyphae (mycelia). In the absence of nutrients, the endoconidia tend to undergo a microcycle of conidiation (production of secondary endoconidia) (Figure 1.5). The latter phenomenon has also been briefly described in two other previous studies (Stover, 1950b; Linderman and Toussoun, 1967).

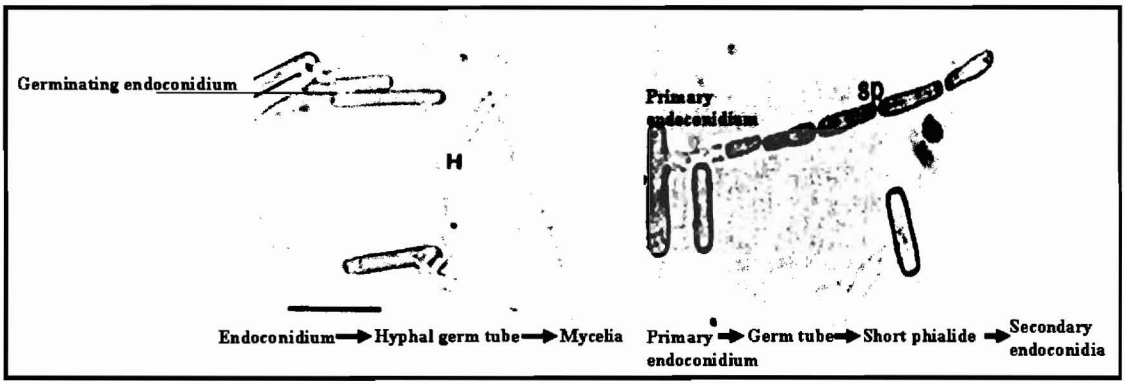

Figure 1.5. Germination in *Thielaviopsis basicola*

In phialospore of *T. basicola*, two types of germination are seen based on the presence or absence of nutrients. In the presence of nutrients, the hyphal form of germination is more dominant. The phialospore produces a germ tube which develops into hyphal germ tubes (H) giving rise to mycelia (left). When nutrients are limited they switch from the hyphal form of germination to produce secondary phialospores (sp) to enhance their chance of survival (right) (Punja 1993).

The distinction in shape between the primary and secondary phialospores has been described by Punja (1993). Secondary endoconidia were smaller in size, had thinner walls

and appeared to be pyriform in shape. Secondary endoconidia production has been considered to occur frequently *in vitro*, however, it has also been described in carrot roots *in vivo* (Punja *et al.*, 1992).

Microcyclic conidiation has also been reported to occur in several other fungi, including *Claviceps* (Bandyopadhyay *et al.*, 1990), *Cercospora* (Fernandez *et al.*, 1991), *Epichloe* (Bacon and Hinton, 1991), *Penicillium* and *Aspergillus* (Smith *et al.*, 1981). Other factors that have been reported to influence microcycle conidiation include germination inhibitors, supra-optimal temperatures and age of the conidium (Smith *et al.*, 1981).

Patrick *et al.* (1965) were able to record the germination process of chlamydopores of *T. basicola* obtained from two sources; a laboratory culture on 2% PDA (potato dextrose agar) and from diseased tobacco plants in the field. They noted that in isolates from both sources the germination process was essentially similar in that in the presence of nutrients, the chlamydospore chains break up into individual cyclindrical cells, each of which may germinate to produce a germ tube which develops into a mycelium. The breaking up of the chlamydospore chains prior to germination suggest that it is a prerequisite for germination (Hawthorne and Tsao, 1969, 1970; Lindsey, 1981). This phenomenon can be accomplished *in vitro* using chitinase treatment or blending (Hawthorne and Tsao, 1970).

Several studies have focused on *in vitro* germination of endoconidia and chlamydospores of *T. basicola* from various hosts. Information collected on the process of germination has proved useful in interpreting data concerning soil fungistasis, spore germination in soil and development of root diseases (pathogenicity) caused by this pathogen. Germination of these two types of spores has also been reported to be affected by temperature, pH, age of culture, spore density, relative humidity, water potential and several other factors (Patrick *et al.*, 1965; Adams and Papavizas, 1969; Hawthorne and Tsao, 1969).

1.9.1.2. Temperature, pH and age of culture

Various studies on the optimum temperature and pH for growth of several isolates of *T. basicola*, show that maximum germination of both endoconidia and chlamydospores of *T. basicola* occurs at temperatures between 20-30°C and pH between 4-8.5 (Lucas, 1955; Mathre and Ravenscroft, 1966). Age of spores of *T. basicola* can also have an affect in the rate of microcycle conidiation under different culture conditions. Germination to produce microcycle conidiation was strain dependent and was greater in older cultures than in younger cultures (Punja, 1993).

1.9.1.3. Spore density, relative humidity and water potential

Brown and Harrower (1997) investigated the effect of spore density, relative humidity and water potential on germination rate of endoconidia of *T. basicola* . They observed the germination rate increased when the spore density decreased and the relative humidity increased. However, when the water potential increased, the germination rate of *T. basicola* endoconidia decreased. Their results demonstrated that spore germination is enhanced as the spore mass is reduced in density. Therefore, they postulated that the dilution of spores leads to the dilution of autoinhibitory substances and reduction in the level of autoinhibitory mechanisms with a concomitant increase in rate of germination of endoconidia until a level is reached below which most endoconidia will germinate. During the conduct of their study, they observed that chlamydospores did not germinate and suggested that their role may be one of long term survival, perhaps under water stress conditions.

1.9.1.4. Growth of *T. basicola* towards host plant roots

Following germination of *T. basicola* spores, the hyphal apex of the germ tube grows towards the surface of its host root hair (Hood and Shew, 1997c, b; Mims *et al.*, 2000). However, research has not yet inverstigated the question of whether the growth of the germ

tube of *T. basicola* is specifically directed towards the host roots or whether growth is a random occurrence. Investigation of this directionality has been one of the objectives of this thesis (see Chapter 2).

Knowledge is only now beginning to emerge on how fungi recognise their hosts. It appears that infection of plants by some fungi is a random process. However, other fungi exhibit considerable selectivity and appear to locate chemical signals derived from the host. This enables pathogenic fungi to form germ tubes and appressoria and to establish intimate relationships with their hosts. Chemical signals include sugars, phenolics and various volatile metabolites released from leaf surfaces (Dean, 1997). The ability of hyphae to change their growth direction is not limited only to nutrient gradients or potential inhibitors but extends to additional external stimuli such as electrical fields (Gow, 1993, 1994), calcium concentration (Gow, 1993, 1994; Lever *et al.*, 1994), pH gradient (Gow, 1993, 1994; Lever *et al.*, 1994) host surface topography (Watts *et al.*, 1998) and ionizing radiation (Zhdanova *et al.*, 2004). Though, much research has looked into fungal germination and growth direction, the environmental signals that induce germination remain largely unknown. These environmental signals are then transferred by signaling genes via signaling cascade pathways to induce a response within the cells. Signaling genes are briefly discussed in section 1.12.5.

1.9.1.5. Contact of *T. basicola* with host root surface

Root hairs are often the host tissue with which hyphae and germ tubes of *T. basicola* makes initial contact (Hood and Shew, 1997b) and epidermal cells are the most common sites of penetration (Hood and Shew, 1996). However, this pathogen has also been reported to enter host roots through non-root hair epidermal cells (Jones, 1991) as well as through wounds (Baard and Laubscher, 1985). The physical contact between *T. basicola* and root hair cells can be inductive (one that initiates infection) or non-inductive. Inductive contact

occurs within minutes from the initial contact between the vegetative hyphal apex (tip of germ tube) and the root hair surface or epidermal cell (Hood and Shew, 1997b; Mims *et al.*, 2000). Upon contact, the hyphal apex discontinues vegetative growth and slightly increases in diameter or swells. This swelling is not regarded as an appressorium as the mechanism of attachment has not yet been confirmed. Active cytoplasmic streaming takes place within the host cell at the site of infection leading to localised accumulation of cytoplasmic components (Figure 1.6) (Hood and Shew, 1997b).

Figure 1.6. Cytoplasmic streaming

Infection of a tobacco root hair by *Thielaviopsis basicola*. The arrow points at the infection site where cytoplasmic streaming is observed 10 minutes after contact. Adapted from Hood and Shew (1997b)

In cases of non-inductive contacts, the vegetative hyphal apex of *T. basicola* continues to grow, redirected by the physical contact of the root hair. However, differentiation of the hyphal apex into infective structures and cytological response within the root hair does not occur. On occasion, non-inductive contacts can lead to inductive contacts (Hood and Shew, 1997b).

1.9.1.6. Penetration of *T. basicola* and establishment of the biotrophic phase

After contact, the swollen hyphal apex develops a slender infection peg (penetration hypha) that penetrates the root hair and protrudes into the cell lumen (Hood and Shew, 1997b; Mims *et al.*, 2000). The host root cell responds by forming papillae but cannot

prevent penetration. A papilla, as observed in a transmission electron micrograph, is characterised as callose deposits present in a central core region containing numerous vesicle-like profiles with an electron transparent outer margin. As the penetration hypha continues to grow through the papilla, its tip expands to form a swollen globose, terminal infection vesicle. This infection structure is believed to be similar to that of a haustorium of the hemibiotrophic leaf pathogen *Entomosporium mespili* in that they both possess a slender neck region with a single septum and an enlarged distal end that contains a single nucleus. After 6 h of contact, infection hyphae emerge from the infection vesicles. The infection hyphae become branched and constrict at their septa giving them a beaded or lance-shaped appearance. The cortical cells of the root are colonized by intracellular lance-shaped infection hyphae (Mims *et al.*, 2000).

The signal to induce penetration does not come from the host because the lumens of dead root hairs can also be penetrated with the hypha eventually exiting the dead cell and resuming vegetative growth. Therefore, it seems that the cytoplasmic components and structural depositions of the host are not responsible for the induction of the penetrating hyphae (Hood and Shew, 1997b).

1.9.1.7. Conversion to necrosis and development of chlamydospores

The generated infection hyphae penetrate and colonize the host cortical cells causing extensive death of cortical cells (necrosis). This is followed by production of several chlamydospores throughout the cortex of the root hairs.

1.10. Nutritional classification of phytopathogenic fungi in living plants

Once a phytopathogenic fungus colonizes a plant, it employs diverse strategies to infect their host plants so as to supply their nutrition and support growth. The major infection strategies include necrotrophy, biotrophy and hemibiotrophy (Prell and Day, 2001).

1.10.1. Necrotrophs

Most necrotrophs kill their host cells by secreting copious amounts of cell wall-degrading enzymes and toxins. Like saprophytes (non-pathogenic fungi unable to kill plant cells), necrotrophs digest the dead plant materials by employing hydrolytic and proteolytic enzymes. The products of digestion are absorbed by the pathogen. *Pythium* and *Botrytis* species are examples of fungal necrotrophs (Prell and Day, 2001). The enzymes responsible for degrading cell walls during necrotrophic fungal growth and have been studied extensively include two classes, the endo-polygalacturonases and the endo-pectin lyases (Bailey, 1991).

Phytotoxins are considered to be one of the major groups of toxins produced by necrotrophic phytopathogenic fungi. Phytotoxins can spread in plants by diffusion from the site of fungal infection to adjacent tissue or through transport via the plant's xylem. Phytotoxins paralyze the plant's defense mechanisms prior to cell killing. There are two classes of phytotoxins, non-host specific (NHS) toxins and host specific (HS) toxins. NHS toxins constitute the major class of phytotoxins. They damage cells of phylogenetically unrelated plants. In contrast, the HS toxins are a smaller class of phytotoxins and only act on host plants carrying genetically determined sensitivity for the particular toxin. They are determinants of pathogenicity factors (exclusively responsible for the appearance of disease symptoms) and host range for some fungi (Prell and Day, 2001). The mechanisms of NHS and HS toxins are described in detail in Prell and Day (2001).

1.10.2. Biotrophs

Biotrophs use more elaborate mechanisms of gaining entry into plant cells, mainly through the formation of appressoria. Some biotrophs, such as *Colletotrichum* species, *Magnaporthe grisea* and others, use the appressoria to directly penetrate the plant cuticle.

However, the majority of biotrophs, such as the rusts and powdery mildews, do not penetrate directly through the cuticle, but instead form apressoria over stomata (Dean, 1997). After entering into the substomatal cavity, the penetration hypha swells and forms a substomatal vesicle. The vesicle continues to elongate and, upon contact with a mesophyll cell, differentiates into a thick-walled haustorial mother cell. A septum is then formed and the haustorium mother cell penetrates the host cell and develops a haustorium, a sophisticated nutrient absorbing apparatus (Dean, 1997). Voegele and Mendgen (2003) showed that haustoria fulfill other biosynthetic functions and may act to suppress plant defenses and to alter the flow of metabolites within plants. Unlike necrotrophs, biotrophs generally have a much narrower host range and do not kill their host plants after infection. Rather they leave the infected cell alive as long as possible because they depend on the unimpaired metabolism of their host cells for nutrition until reproduction of the pathogen is completed. This results in delayed disease symptoms in the host plant, which appear long after infection offering the plant the opportunity to raise new defense mechanisms (Prell and Day, 2001).

1.10.3. Hemibiotrophs

Hemibiotrophs are parasites that require living host cells during part of their life cycles, and develop specialized structures during infection that are not found during axenic growth (Kahmann and Basse, 2001). They differ from biotrophs by the fact that in later stages following infection, they become necrotrophic and typically sporulate on dead tissues of their host (Luttrell, 1994). That is, they must first pass through a biotrophic phase and later, after the breakdown of host plant tissue, switch to a necrotrophic phase (Prell and Day, 2001). The length of the biotrophic phase exhibited by hemibiotrophs appears to vary greatly (Parbery, 1996). According to Parbery (1996), hemibiotrophs can be divided into biotrophic hemibiotrophs and necrotrophic hemibiotrophs. Biotrophic hemibiotrophs include species in which the death of host cells is delayed until about the time the pathogen

begins to sporulate. On the other hand, necrotrophic hemibiotrophs have a short biotrophic phase that allows them to take possession of host tissues before killing and invading the tissue. Therefore, the main difference that exists in the hemibiotrophs is the duration of the bitrophic phase.

1.10.4. *T. basicola* as a hemibiotroph

T. basicola was initially considered to be a saprophyte or a weak facultative parasite, implying the ability to survive long periods in the absence of a host by saprophytic utilization of soil-borne organic matter (Garrett, 1956). However, Mims *et al.* (2000), using transmission electron microscopy (TEM) provided a more detailed description of the way in which this important hemibiotrophic pathogen attacks its host, in this case pansy roots, and the ways in which host roots respond to penetration and infection. Their results showed consistency with Hood and Shew (1997b) in that *T. basicola* fits the description of a hemibiotroph since it establishes a biotrophic phase before a necrotrophic phase. However, controversy exists between both studies as of which type of hemibiotroph this pathogen belongs to. Whereas, Hood and Shew (1997b) observed that in black root rot on tobacco the biotrophic phase lasted for days, Mims *et al.* (2000) observed that host epidermal cell necrosis began in invaded pansy root as early as 8 h post inoculum (PI) and that extensive death of cortical cells had occurred within 24 h. Therefore, Mims *et al.* (2000) classified *T. basicola* as a "necrotroph" hemibiotroph.

1.11. Control of black root rot

1.11.1. Cultural control

To help minimize black root rot of cotton, various cultural methods have been employed. The agronomic benefits of using crop rotations, summer flooding, exclusion, timed planting and sanitation practices have been documented (Nehl *et al.*, 2000). Rotation with

non-host crops, in particular cereals, has shown the potential to reduce inoculum levels of *T. basicola* in the soil.

Research has shown that inclusion of a hairy vetch (*Vicia villosa*) as a cover crop in rotation with cotton decreased inoculum levels of *T. basicola* and the percentage of seedling colonization. Furthermore, hairy vetch amended soils significantly suppressed populations of *T. basicola* and decreased black root rot of cotton compared to nonamended soils (Kendig and Rothrock, 1991). Candole and Rothrock (1998) have assessed *T. basicola* suppression by means of marked strains (benomyl-tolerant *T. basicola* strains) in hairy vetch amended soils. The effect of hairy vetch amendment decreased the populations of benomyl-tolerant *T. basicola* from diseased roots of cotton seedlings due to the presence of low amounts of ammonia in hairy vetch amended soils (Candole and Rothrock, 1997, 1998).

Glasshouse experiments in the USA indicated that flooding decreased the severity of black root rot substantially for up to four seasons. Flooding was most effective during summer and required maximum air temperatures of 30°C or more for at least 30 days. Although, flooding in Australia has also been proven to be effective, however, its adaptation as a tool to control disease is restricted by the availability of water (Nehl *et al.*, 2000)

Timed planting of crops such as cotton should be planned to coincide with higher temperatures (above 16°C) as cool temperatures favour black root rot of cotton. For example, crops that were sown in high temperatures appeared to be stunted with black root rot but had passed the stage of being susceptible to *Rhizoctonia* and appeared more healthy than crops that were sown in cool temperatures (Nehl *et al.*, 2000).

Sanitation practices are important in preventing the spread of *T. basicola*. Since it is suspected that some cotton isolates of *T. basicola* might have been originally introduced

into Australia via imported second-hand ginning equipment from California which was contaminated with infested soil, ensuring that equipment moved from farm to farm is inoculum free is extremely important for controlling the spread of black root rot (Honess, 1994).

1.11.2. Chemical control

Fungicides are an important tool in managing root rot diseases when used in conjunction with cultural management practices. Fungicides rarely, if ever, eradicate fungal pathogens; they merely suppress disease development for a certain period of time. If conditions for fungal infection are favorable after this period, the disease may once again progress (Honess, 1994).

Several soil fumigants have been widely used to control some crops such as tobacco, with some fumigants being more effective than others (Ontario, 1995). Generally, the effectiveness of fumigations depends on the fumigant used and the environmental conditions present at the time of fumigation (Haji and Brandle, 2001). Fumigants are injected into the soil (15 cm deep) three weeks before sowing or transplanting a crop (Gayed, 1967). The application of conventional soil fumigation to cotton diseases in Australia is not a practical option because of the scale of production (434,000 ha in 1997/1998). The cost of the usage of fumigants on cotton would not justify the return. In addition, fumigants fail to disperse and penetrate adequately due to the high clay content in many of the soils used to grow cotton. Moreover, fumigants could cause a negative environmental impact (Matthiessen and Kirkegaard, 1999). Studies have also reported that control of cotton disease pathogens depends to some extent on planting high quality seeds that are properly treated with fungicides. In preliminary greenhouse and field trials, various fungicide seed treatments reduced the severity of black root rot and increased seedling survival (Minton *et al.*, 1982).

Exclusion of the pathogen is recommended, as prevention is better than the cure. Using Castrol Farmcleanse at 10% will kill the black root rot fungus and aid in the decontamination of vehicles used in farming so as to minimize the spread of *T. basicola* among fields, farms and valleys (Rourke and Nehl, 2001).

1.11.3. Biocontrol

The active suppression of soil-borne pathogens by biocidal compounds is known as biofumigation (Matthiessen and Kirkegaard, 2006). This method involves planting a 'green manure' crop that releases compounds that are toxic to pests or pathogens in the soil (Nehl *et al.*, 2000). One of the principle biocidal compounds is isothiocyanate (ITC) produced by the enzymatic hydrolysis of glucosinolates in cruciferous plants. The main ITC, 2-phenylethyl ITC (2-PE ITC), liberated from the roots of canola, was tested *in vitro* and found to exhibit an inhibitory effect on *T. basicola* as well as other fungi (Smith and Kirkegaard 2002). Like fungicides, biofumigation does not eradicate *T. basicola.* In Australia, the biofumigants, CSIRO Indian mustard line 651, Peterson White Mustard Maxi and Wooly pod vetch have succeeded in reducing the severity of black root rot in cotton by approximately 60% (Matthiessen and Kirkegaard, 1999). A biocontrol agent *Trichoderma virens* may also be effective in controlling black root rot of cotton, as it has been shown to induce terpenoid synthesis, a defense response in cotton roots infected with the root pathogen *Rhizoctonia solani* (Howell *et al.*, 2000; Howell, 2003).

Putrescine, a low molecular weight compound produced in cells of animals, plants, bacteria and fungi has been found to play a role in cell growth and differentiation. However, high levels of putrescine in burley tobacco have been shown to inhibit the reproduction of several fungi, including *T. basicola.* The mechanism of the fungicidal effect of putrescine on *T. basicola* is still unknown (Harrison and Shew, 2001). Another biocontrol agent that is active against several soil-borne fungal plant pathogens and shows

antifungal activity in culture assays is *Pseudomonas aureofaciens* strain 63-28. This bacterium produces a compound called 3-(1-Hexenyl)-5-methyl-2-(5H) furanone, which has been shown to have antifungal activity against *Pythium ultimum*, *Fusarium solani*, *F. oxysporum* and *T. basicola*. This compound is also similar in structure to other antifungal furanones produced by actinmoycetes (*Streptomyces* species), fungi (*Trichoderma harzanium*) and higher plants (*Pulsatilla* and *Raniculus* species) (Paulitz *et al.*, 2000).

1.11.4. Breeding and genetic manipulation of plants

Disease management by genetic control is expected to be the primary means of controlling black root rot. Natural host genetic resistance has mainly been implicated in the control of black root rot of burley tobacco (*Nicotiana tabacum*). The type of resistance exhibited by wild-type tobacco genomes includes only polygenic partial resistance to black root rot. Complete resistance to a wide spectrum of black root rot isolates has been found to exist in a tetraploid relative of tobacco, *Nicotiana debneyi* Domin. A single dominant gene controls this resistance and has been successfully transferred to burley tobacco by backcrossing (Shew and Shoemaker, 1993; Bai *et al.*, 1996; Hood and Shew, 1996). Tobacco lines carrying *N. debneyi* chromosomes with a gene for resistance to black root rot have been produced and characterised (Bai *et al.*, 1996).

Research has also looked into host plant resistance in combination with fumigant treatments containing chloropicrin (CP) to assess the resistance to black root rot. A recent study on the performance of resistant tobacco cultivars (controlled by a single dominant gene) with and without fumigation treatments has been conducted in Ontario, Canada. Their results suggested that *T. basicola* resistant cultivars can be used to manage black root rot grown in Ontario without the use of chloropicrin as a soil fumigant. However, the application of the soil fumigant, Vorlex Plus without chloropicrin, could reduce black root rot population depending on soil conditions (Haji and Brandle, 2001).

Screening programmes have also been initiated to screen cotton germ plasm for resistance to *T. basicola.* One diploid cotton line (*Gossypium arboretum*, PI 1415) with an apparent high level of resistance to black root rot has been identified. Incorporation of the resistance factor PI 1415 into tetraploid upland cotton, especially in combination with fungicide seed treatments could be used as a strategy to greatly reduce damage by black root rot (Wheeler *et al.*, 1999).

Ways to activate these inherent disease resistant mechanisms of plants have recently been the focus of research by plant pathologists. The application of synthetic chemical inducers to the plant, in advance of the pathogen (in the absence of the pathogen) has reduced the severity of black root rot in cotton and legumes (Nehl *et al.*, 2000)

Transgenic plants are now being developed to acquire high levels of resistance against fungal pathogens. Research on the genetic mechanisms employed by the biocontrol agent *Tricoderma virens* has revealed a gene (Gv29-8) that encodes an endochitinase, which when engineered into cotton demonstrated high levels of resistance to *T. basicola* (Howell, 2003).

Bioactive peptides with potential for control of fungal pathogens have also been investigated with the ultimate goal of identifying candidates with which to genetically engineer cotton. The findings of Kristyanne *et al.* (1997) revealed that magainin 2, a 23-amino acid isoform from the African clawed frog, has broad-spectrum antimicrobial activity that inhibits the growth of fungi. It completely inhibited hyphal growth of some plant pathogens, including *T. basicola,* in culture (Kristyanne *et al.*, 1997). Further studies of magainin, recently conducted by Chakrabarti *et al.* (2003), showed that its analogue MSI-99, conferred enhanced disease resistance to fungal pathogens in transgenic plants when subcloned into plant expression vectors and transformed into banana and tobacco plants. In Australia, the utilization of DNA marker technology to select cotton varieties

carrying different genes for disease resistance could help plant breeders to pyramid several combinations of such genes in the one plant variety. This technology holds much promise in producing varieties with higher disease tolerance to a range of microbial pathogens (Lyon *et al.*, 2000). It is hoped that an improved understanding of the genetic factors responsible for the pathogenicity of *T. basicola* will uncover novel approaches for disease control.

1.12. Fungal pathogenicity genes

The economic importance of fungal phytopathogens has stimulated research into the molecular genetics of fungal pathogenesis. The application of molecular techniques such as random and targeted insertional mutagenesis is shedding light on the mechanisms of fungal pathogenicity. Understanding mechanisms of fungal pathogenicity and the genes that control them may allow the use of recombinant DNA technology to modify the plant's defense system to induce resistance to attack. Disruption of a pathogenicity gene results in the reduction or complete loss of disease symptoms. Fungal pathogenicity genes have been a subject of several reviews over the last decade (Schafer, 1994; Dean, 1997; Idnurm and Howlett, 2001; Tucker and Talbot, 2001; Tudzynski and Sharon, 2003). Idnurm and Howlett (2001) have defined pathogenicity genes as genes necessary for disease development, but not essential for the pathogen to complete its life cycle *in vitro*. The types of genes essential for pathogenesis depend on the infection process of a particular fungus. For a comprehensive review of fungal pathogenicity genes, Idnurm and Howlett (2001) tabulated 79 pathogenicity genes identified since 1990. The majority of pathogenicity genes enable the fungus to establish infection, and these include genes that encode proteins with specific roles in formation of infection structures, cuticle and cell wall degradation and the synthesis of toxins. However, a number of pathogenicity genes are involved in responding to the host environment in order to defeat the plant defense

system. All of these genes are connected with signaling genes. The intention in this section is not to provide a complete list of pathogenicity genes, but only to provide some example of genes in each category mentioned above.

1.12.1. Formation of infection structures

Genes involved in the formation, development and maintenance of infection structures such as an appressorium include genes that play a role in melanization, conidiation and recognition of the host surface. Melanin, a dark brown to black pigment, produced by many orders of fungi, including the Ascomycetes, is derived via the polyketide pathway (Gomez and Nosanchuk, 2003). One of the crucial roles of melanin in phytopathogenic fungi is to prevent the leakage of glycerol from the appressorial wall. The presence of glycerol is known to provide turgor pressure enabling the appressoria to puncture the plant epidermis (Mendgen *et al.*, 1996). Melanin-deficient mutants in *M. grisea* are unable to provide turgor pressure and are non-pathogenic. However, they retain the ability to differentiate an appressorium and a penetration peg that can invade wounded leaves but not intact plant surfaces (Chumley and Valent, 1990). In another study of *M. grisea*, the targeted deletion of *PLS1* (a gene encoding a tetraspanin-like protein) generated mutants that were unable to invade wounded leaves suggesting that it lacks the ability of penetration peg differentiation (Clergeot *et al.*, 2001).

Mutations of *mpg1* (a hydrophobin that plays a role in attachment and conidiation) reduces pathogenicty and mutations in *ACR1* (acropetal) genes in *M. grisea* led to defective conidiation and a loss of pathogenicity due to impaired appressorium formation (Lau and Hamer, 1996). The disruption of another gene, *pth11* in *M. grisea*, which encodes a cell membrane protein, resulted in non-pathogenic mutants unable to recognise the host surface (DeZwaan *et al.*, 1999). In soil-borne plant pathogens, genes involved in appressorium formation are less understood because penetration of roots occurs without an appressorium

(Di Pietro *et al.*, 2003). *T. basicola* is not known to produce appressoria or other specialized infection structures (Hood and Shew, 1997b).

1.12.2. Cuticle and cell wall degradation

Phytopathogenic fungi that invade by direct penetration through the plant surface have to breakthrough the defense barriers such as the cuticle and cell wall. This penetration is achieved by enzymatic degradation. The disruption or deletion of endopolygalacturonase genes, *Bcpg1* in *Botrytis cinerea* (ten Have *et al.*, 1998), *pecA* in *Aspergillus flavus* (Shieh *et al.*, 1997), and *Acpg1* in *Alternaria citri* (Isshiki *et al.*, 2001) resulted in reduced lesions on leaves and fruit of tomato and apple fruit, cotton bolls, and on citrus fruit respectively. Recent disruption studies on the cutinase gene of *Pyrenopeziz brassicae*, an ascomycete, showed molecular evidence that cutinase is required for pathogenicity (Li *et al.*, 2003). However, previous cutinase disruption studies with other fungi for example *F. solani* (Stahl and Schafer, 1992), *M. grisea* (Sweigard *et al.*, 1992) and *B. cinerea* (van Kan *et al.*, 1997) have produced conflicting evidence, suggesting that cutinase is not always required for fungal pathogenicity. The question whether a single enzyme involved in cuticle and cell wall degradation can play an essential role in pathogenicity has been difficult to prove in some studies. It is suggested that enzymes act synergistically during cuticle and cell wall degradation and the loss of a single activity may not cause a detectable change in plant infection and colonization (Schafer, 1994). Therefore, disruption of the function of several extracellular enzymes in a particular transformant might be needed to abolish or reduce pathogenicity. For example, disruption of the pectate lyase genes, *pelA* or *pelD*, in *F. solani f. sp. pisi* generated transformants that were still pathogenic, however, when both genes were disrupted, *F. solani* mutants showed reduced pathogenicity on pea (Rogers *et al.*, 2000). Disruption of *ccsnf1* in the maize pathogen *Cochliobolus carbonum* encoding a regulator of catabolite-repressed genes, reduced the transcription of 10 cell wall degrading enzymes, resulting in mutants with reduced pathogenicity (Tonukari *et al.*, 2000). To date

there have been no studies conducted to investigate whether *T. basicola* generates enzymes that degrade plant cell walls and other physical barriers.

1.12.3. Responding to host environment

Inside the host tissue, the fungus encounters plant chemical defences such as anticipins and phytoalexins. It is also subjected to nutrient limitation. One of the resistance mechanisms employed by plant pathogenic fungi is the secretion of enzymes that degrade or detoxify these plant chemical defenses. For example, targeted disruption of the avenacinase gene in the fungus *Gaeumannomyces graminis* var. *avenae* prevented mutants from attacking oat roots that produce *avenacin A1* (Bowyer *et al.*, 1995). The detoxification of phytoalexins was shown in the fungus, *Nectria hematococca* by VanEtten and coworkers (1989). Disruption of *PDA1*, a gene that encodes an enzyme pisatin demethylase that degrades the pea phytoalexin pisatin, resulted in only a small decrease in disease severity (Wasmann and VanEtten, 1996). Loss of an indispensable chromosome where the *PDA1* gene is located led to greater reduction in pathogenicity implying that there were other pathogenicity genes adjacent to *PDA1* (VanEtten *et al.*, 1994).

At some stage during infection, the availability of nutrients for the fungus is low. Whether nutrient limitation plays a role in pathogenicity has been demonstrated using gene disruption studies and has been induced during *in vitro* starvation conditions. Mutational analyses have shown that starvation-induced genes are essential for pathogenicity in leaf and root invading fungi (Idnurm and Howlett, 2001). It was found that the regulation of certain compounds associated with, for example, nitrogen assimilation is important for disease development by phytopathogenic fungi on leaves and roots but not necessary for pathogenicity. For example, *NUT1* (a gene homologous to the major nitrogen regulatory genes *nit-2* of *N. crassa* and *areA* of *Aspergillus nidulans*) mutants of *M. grisea* failed to grow on a variety of nitrogen sources. The *NUT1* mutants showed similar infection

efficiency to colonise susceptible rice plants to that of the parental strain, however, lesions were reduced in size (Froeliger and Carpenter, 1996). To date the response of *T. basicola* to *in vitro* starvation conditions has not been investigated.

1.12.4. Fungal toxins

As described in section 1.10.1, fungal toxins include NHS and HS toxins. NHS toxins include the tricothescenes produced by various *Fusarium* sp (Brown *et al.*, 2001). Disruption of genes involved in the biosynthesis of trichothescenes have also resulted in reduced pathogenicity of *Gibberella pulicaris* on parsnip (Desjardins *et al.*, 1992) and *G. zeae* on wheat (Proctor *et al.*, 1995).

Fungi with a limited host range are characterised by the production of HS toxins. About 20 HS toxins have been documented of which at least nine are from *Alternaria alternata* pathotypes (Walton, 1996). *A. alternata*, apple pathotype, produces the HS toxin AM-toxin causing Alternaria blotch on a narrow range of susceptible apple cultivars. Disruption of genes involved in the biosynthesis of AM-toxin (by protoplast transformation) resulted in toxin-minus mutants which were unable to cause disease symptoms (Johnson *et al.*, 2000). It has also been reported that the genes that control biosynthesis of HS toxins are often clustered, but not all genes in the cluster are involved in toxin biosynthesis, and that a fungus with an altered toxin profile can still cause disease. For example, genes that are involved in the biosynthesis of HC-toxin, a virulence and specificity factor in *Cochliobolus carbonum*-maize interaction, are clustered forming a giant locus (*TOX2*) spanning 600 kilobases that include repeats of at least 6 genes. *HTS1* (encodes a peptide synthetase), *TOXC* (encodes a fatty acid synthase beta subunit) and *TOXF* (encodes an amino acid transaminase) have been reported to be essential for HC-toxin biosynthesis and pathogenicity. However, the disruption of *TOXG* (provides a $_{D}$-isomer of alanine for HC-

toxin) or TOXEp (a regulatory protein) did not show reduced pathogenicity (Ahn *et al.*, 2002). So far, there have been no reports of toxins generated by *T. basicola*.

1.12.5. Signaling genes

Signaling cascades are used by fungi to alter their gene expression in response to changes in the environment. Genes involved in signaling cascades have been studied in several pathogenic fungi focusing on heterotrimeric G proteins, mitogen activated protein kinase (MAPK) and the cyclic AMP-dependent protein kinase (cAMP-PK) genes. Such genes constitute the majority of fungal pathogenicity genes (Tudzynski and Sharon, 2003). It is hoped that the components of these signal transduction pathways could serve as targets for the development of antifungal drugs. The disruption of these signaling genes has been shown to cause reduced or loss of pathogenicity as well as toxin production, mating, growth rate and conidiation (Idnurm and Howlett, 2001). Signaling genes have been the subject of several reviews (Dean, 1997; Idnurm and Howlett, 2001; Tucker and Talbot, 2001; Tudzynski and Sharon, 2003). MAP kinases are essential for pathogenicity of leaf infecting fungi such as *M. grisea*, *Cochliobolus. heterostrophus* (Lev *et al.*, 1999), *Colletotrichum lagenarium* (Takano *et al.*, 2000) and *B. cinerea* (Zheng *et al.*, 2000), however, their role in other plant tissues is less understood. Recently, Dufresne and Osbourne (2001) have demonstrated using mutational analyses that the MAP kinase pathway also plays a role in infection of plant root tissues by *M. grisea*, and are therefore considered to be global regulators of fungal pathogenesis. Di Pietro *et al.* (2001) provided evidence using transformation mediated targeting that the *fmk1* gene of *F. oxysporum*, which encodes a MAPK gene plays an essential role in root penetration. The targeted inactivation of *fmk1* gene encoding encoding a MAPK in *F. oxysporum* were found to be unable to attach to tomato roots and therefore were unable to penetrate and cause any disease symptoms. The *fmk1* mutants also showed decreased production of polygalacturonases and pectate lyases that participate in the degradation of the plant cell

wall (Di Pietro *et al.*, 2001). The targeted inactivation of the gene *fga1* encoding a Gα subunit in *F. oxysporum* resulted in altered colony morphology, reduced conidiation, increased heat resistance and reduced pathogenicity (Jain *et al.*, 2002). The plieotropic effects shown in the *fga1* or *fmk1 F. oxysporum* mutants implicate an essential role of these genes in different signaling pathways regulated by the Gα subunit or the MAPK pathways respectively.

1.12.6. Novel and unclassified pathogenicity genes

Several other novel pathogenicity genes have been correlated to pathogenicity and these include genes that either have no homologues in gene databases or reveal new mechanisms on the plant-pathogen interaction. For example, in the root invading fungus *F. oxysporum, FOW1* (a gene that encodes a mitochondrion carrier) mutants showed markedly reduced virulence as a result of a defect in the ability to colonise the plant tissue and provided evidence that a specific mitochondrial protein may play a role in fungal pathogenesis (Inoue *et al.*, 2002). *CLTA1* (a GAL4-like transcription activator) from the bean pathogen *C. lindemuthianum* was also found to be involved in the colonization of host tissue (Dufresne *et al.*, 2000). To date, no studies on mutational analyses using insertional mutagenesis of *T. basicola* have been reported. Therefore, the development of molecular biology tools is essential for the identification of *T. basicola* pathogenicity genes.

1.13. General aims of this study

This study is part of a multidisciplinary research approach aimed at the identification of pathogenicity factors involved in the interaction of *T. basicola* and its host plants leading to black root rot. The identification of such factors will enable other studies to analyse the function of the identified factors in their influence or control of the interaction of *T. basicola* and its hosts. Thereon, strategies can be developed to interrupt or block various stages of the infection process leading to black root rot and thus reduce the severity of the disease.

The main objectives of this study were to investigate *T. basicola* host specificity, to develop a genetic transformation protocol for *T. basicola* in order to generate mutants which are altered in their ability to establish host infection and to identify the gene/s affected in these mutants.

Chapter 2 . Pathogenicity and directional growth of *T. basicola* isolates towards various plants

2.1. Introduction

In order for *T. basicola* to cause black root rot, it differentiates into infection structures and enters the plant cells eventually causing necrosis. This involves the germination of *T. basicola* in soil, emergence and elongation of the germ tube, reaching the plant roots, attachment to the root surface and penetration into the root hairs or epidermal cells to start the biotrophic phase. Evidence of host specificity in *T. basicola* has been reported previously (Keller and Shanks, 1955; Lloyd and Lockwood, 1963; Tabachnik *et al.*, 1979; O'Brien and Davis, 1994; Bai *et al.*, 1996). If signaling molecules are involved in the infection process, a specific interaction between the pathogen and host probably exists. It is possible that pathogen-host specificity could be expressed during several steps of the infection process. Two aspects of host specificity in the infection process involve directional growth and the ability to cause disease once the fungus comes into contact with the host.

Because *T. basicola* is a soil-borne pathogen, roots play an important role in the infection process by producing a wide variety of compounds, which may serve as stimulants, attractants, inhibitors, or genetic regulatory signals for many pathogenic fungi prior to colonization. In the soil, diffusion of root exudates can provide a concentration gradient, thus allowing the fungus to locate the root. The germination of spores of *T. basicola* in response to root extracts (Patrick *et al.*, 1965), root exudates (Mathre *et al.*, 1966) and some specific stimulatory substances (Papavizas and Adams, 1969) has been well documented. However, the question as to whether the growth of the hyphae is specifically directed towards the host root, possibly due to a specific host signal, or whether hyphal

growth is random and comes into contact with the host by chance has not been previously investigated.

The objectives of this chapter were to investigate the ability of *T. basicola* isolates to perceive possible signals generated by various plants and respond by directed growth to them as well as to analyse the susceptibility of various plants to direct inoculation. The hypothesis put forward in this study is that nutrient gradients that exist around plant roots are responsible for *T. basicola* hyphal directional growth towards the root surface of the plant. This could be important for analyzing *T. basicola* mutants affected in hyphal directional growth and pathogenicity towards susceptible host plants. Furthermore, this could also lead to future identification of a host specific stimulus that initiates or causes spore germination, hyphal growth direction and virulence that is fundamental and could be exploited by breeding cultivars with reduced stimulatory effect.

2.2. Materials and Methods

2.2.1. Culture conditions for *Thielaviopsis* isolates

Isolates of *T. basicola* from several hosts and one isolate of *Thielaviopsis thielavioides* from carrot were obtained from culture collections. Stock cultures of *Thielaviopsis* isolates were maintained on agar blocks in sterile distilled water (DW) and subcultured every 6 months on ½ strength potato dextrose agar (½ PDA) containing 2.2% agar (2.2.2.1). Working cultures were maintained on ½ PDA (2.2% agar) and stored at 4°C until needed. Table 2.1 and Table 2.2 below show the *Thielaviopsis* isolates and plants used in this study.

Table 2.1. *Thielaviopsis* isolates

Thielaviopsis Isolate	Accession number	Obtained from
Cotton	BRIP40192	Jan Dean, Dept. of Primary Industries, QLD govt.
Lettuce 1	BRIP40191	Jan Dean, Dept. of Primary Industries, QLD govt.
Lettuce 2	UQ4989	John Harvey, UQ
Carrot	5247-6	John Harvey, UQ
Carrot BT[a]	Carrot BT	John Harvey, UQ
Lupin	JHA21	John Harvey, UQ
Pansy	03185	John Harvey, UQ

[a] *Thielaviopsis thielavioides*, all others were *Thielaviopsis basicola* isolates

Table 2.2. Plants exposed to *Thielaviopsis* isolates

Plant	Scientific name of plant	Cultivar	Obtained from
Cotton	*Gossypium hirsutum*	SICOT 189 BR	Narrabri
Lettuce	*Lactuca sativa*	Cos Lobjotis	Terranova
Carrot	*Daucus carota*	All season	Krempin's seeds
Lupin SA	*Lupinus angustifolius*	Wonga	"Alton Park", Dubbo, NSW
Pansy	*Viola* sp. cult.	Pansy clear crystals	Krempin's seeds
Durm Wheat	*Triticum durum*	Wollaroi	UNE
Broccoli	*Brassica oleracea*	Green sprouting	Krempin's seeds
Rice	*Oryza sativa*	Jarrah	UNE

2.2.2. Growth media

2.2.2.1. ½ PDA (2.2% agar)

½ strength potato dextrose agar contained 1.95% (w/v) potato dextrose agar (Oxoid Ltd., Hampshire, England) and 1.45% (w/v) agar (Oxoid).

2.2.2.2. Water agar (1.2%)

Water agar contained 1.2% (w/v) agar (Oxoid)

2.2.2.3. Noble agar (1.2%)

Noble agar contained 1.2% (w/v) Noble agar (DIFCO)

2.2.2.4. Yeast mannitol agar (YMA)

Yeast mannitol agar contained 0.1% (w/v) Yeast extract, 1% (w/v) Mannitol, 0.05% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_47H_2O$ and 1.5% (w/v) agar (Oxoid).

2.2.3. Inoculum production

The endoconidia used for the experiments were obtained from 5 days old *Thielaviopsis* cultures grown on ½ PDA (2.2% agar) under a 12 h light/12 h dark cycle at 25°C. Spore suspensions were prepared by scraping mycelia from ½ PDA (2.2% agar) plates into sterile McCartney bottles containing 7 mL of sterile de-ionised water, and vortexing the suspension at high speed for 1 min. The spore suspensions of *Thielaviopsis* isolates were filtered through one layer of dry Miracloth (Calbiochem) (pre-soaked overnight in 70% ethanol) to separate chlamydospores and mycelia from endoconidia. The number of endoconidia was estimated using a haemocytometer, and was then adjusted with sterile de-ionised water to 3.5×10^5 endoconidia/mL. This concentration was used for pathogenicity tests (section 2.2.5), directional growth tests (section 2.2.7) and host preference tests (section 2.2.7).

2.2.4. Seed sterilization and seed weight

Different surface sterilization protocols were used for the various seeds depending on the size of the seed. Cotton, lupin, wheat and rice seeds were surface sterilized in 0.5% mercuric chloride for 5 min. Lettuce, pansy, carrot and broccoli seeds were surface sterilized using 10% ethanol and 4% sodium hypochlorite for 1 min. Following surface sterilization, seeds were rinsed five times in sterile de-ionised water. For germination and detection of contamination, surface sterilized seeds were placed on YMA (section 2.2.2.4). For determining the weight of seeds, a random selection of seeds was weighed. The weight in grams of seeds was recorded from three replicates.

2.2.5. Pathogenicity tests

The pathogenicity assays were performed following a modified procedure of Nan *et al.* (1992). Surface sterilized seedlings (cotton, lupin, lettuce, carrot, pansy, broccoli, wheat and rice) were germinated on YMA. Germinated seedlings with radicles of uniform length (1 cm) free from microbial contamination were collected and dipped for 1 min in 5 mL of *Thielaviopsis* endoconidial suspensions (3.5×10^5 spores/mL) of each *Thielaviopsis* isolate. Control seedlings were dipped in the same batch of sterile de-ionised water without endoconidia. Each inoculated or control seedling was then transferred individually to water agar plates (1.2% agar) (55 ×14 mm) and incubated at 25°C in a 12 h light/12 h dark cycle. Seven days after inoculation, roots were examined for lesions and the lesions were examined under the stereomicroscope for the presence of chlamydospores. The length of root lesions as well as the total root length was measured with a ruler. Disease severity was expressed as the percentage of the total root length affected by the lesion. Necrotic lesions induced by *Thielaviopsis* isolates on roots of various plants susceptible or non-susceptible to black root rot were rated as shown in Table 2.3.

Table 2.3. Rating of necrotic lesions induced by *Thielaviopsis* isolates on roots of various plants susceptible or non-susceptible to black root rot

Necrotic root lesion (%)	Rating	Reaction to black root rot
0-20	Non-pathogenic	Non-susceptible
21-40	Weakly virulent	Non-susceptible
41-60	Intermediate in virulence	Susceptible
61-100	Highly pathogenic	Susceptible

The experiments were performed three times with five replicates of each *Thielaviopsis* isolate per experiment.

2.2.6. Growth rate of *Thielaviopsis* isolates

To determine the growth rate of *Thielaviopsis* isolates, a sterilized inoculating needle was used to gently scrape spores from five-day-old cultures on ½ PDA (2.2% agar) plates and stabbed in the center of fresh ½ PDA (2.2% agar) and water (1.2% agar) plates. After 7 days of incubation at 25°C in a 12 h light/12 h dark cycle the colony diameter (in cm) was measured. Three replicates were used for each *Thielaviopsis* isolate.

2.2.7. Growth direction and host preference tests

The growth directionality and host preference of *Thielaviopsis* isolates when exposed to germinating seeds of cotton, lupin, lettuce, broccoli, wheat or rice were tested using 1.2% Noble agar on either round plates (90 × 14 mm, vented) or square plates (100 × 100 × 2 mm, without vents), respectively. A volume of 70 µL of endoconidial suspension (3.5 × 10^5 endoconidia/mL) of each *Thielaviopsis* isolate was absorbed onto a strip of sterile filter paper (6.5 × 1 cm) (Whatman no. 1 chromatography paper). For growth directionality tests, endoconidial strips were placed directly onto Noble agar plates 2 cm away from four surface sterilized seeds (Figure 2.1 A). For host preference tests, endoconidial strips were placed at the center of Noble agar plates and exposed simultaneously to two types of seeds. Several surface sterilized seeds were placed 2 cm away from the spore strip (Figure 2.1 B).

In both growth direction and host preference tests, the surface sterilized seeds were spread out evenly in a straight line along the plates. Control treatments consisted of two sterile filter paper strips (6.5 × 1 cm) (Whatman no. 1 chromatography paper); one contained a volume of 70 µL of endoconidial suspension (3.5×10^5 endoconidia/mL) of each *Thielaviopsis* isolate and another contained 70 µL of sterile de-ionised water.

All plates were incubated for 7 days at 25°C in a 12 h light/12 h dark cycle. Roots of seedlings that reached approximately 2 cm were replaced with fresh surface sterilized seeds. Seven days after inoculation, mycelial growth on all 1.2% Noble agar plates were examined under the stereomicroscope to confirm the presence of *Thielaviopsis* chlamydospores and mycelia. Growth directionality results (raw data not shown) were assessed by measuring the distance of the mycelial growth from the side of the strip towards the seedlings and subtracting the distance of the mycelial growth away from the seedlings (Figure 2.1 A). Host preference results were assessed by measuring and comparing the length of mycelia on each side of the spore strip towards the different seedlings (Figure 2.1 B). The experiments were performed three times with five replicates of each *Thielaviopsis* isolate per experiment in both directional growth and host preference tests.

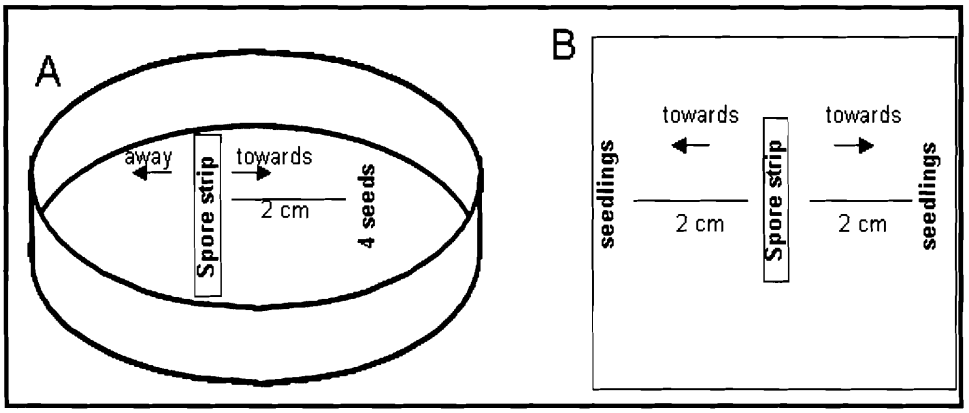

Figure 2.1. Assessment of growth direction and host preference tests.

(A). Growth direction tests. Growth direction plates were assessed by measuring the distance of mycelial growth towards the seedlings (for example 1.5 cm in length) and subtracting the distance of mycelial growth away from the seedlings (for example 0.5 cm in length).

(B). Host preference tests: Host preference plates were assessed by measuring and comparing the length of mycelia on each side of the spore strip towards the seedlings. Mycelial growths on all plates were examined under the stereomicroscope to confirm the presence of *Thielaviopsis* chlamydospores and mycelia.

The directional growth of *Thielaviopsis* isolates induced by roots of various plants susceptible or non-susceptible to black root rot were rated as shown below in Table 2.4.

Table 2.4. Rating of directional growth of *Thielaviopsis* isolates induced by roots of various plants susceptible or non-susceptible to black root rot

Hyphal directional growth (cm)	Rating of directional growth
0-0.25	Weak
0.26-0.5	Intermediate
0.6-1	Strong

The raw directional growth data (data not shown) for each isolate was adjusted by dividing the directional growth data for each isolate by the relative growth rate for each isolate. The relative growth rate for each isolate was obtained by dividing the growth rate for that isolate on ½ PDA (2.2% agar) by the total mean growth rate of all isolates. This adjustment was done so that differences in directional growth rate of the isolates towards the seeds were not only due to inherent differences in growth rate between the isolates but also to differences in the response of the isolates to stimulation by the seeds.

2.2.8. Statistical analysis

Each plate in all experiments described in this chapter was treated as a replicate, and data from the experiments were averaged and subjected to ANOVA using SPSS (SPSS Inc. Chicago, USA). When treatment effects were significant, Duncan's multiple range test was used to identify treatments that were significantly different from each other.

For pathogenicity and growth directionality tests, ANOVA showed that there was no interaction between experiment and isolate for any plant species. The three experiments for each plant species were therefore combined for analysis, with each experiment treated as a block. Because the different plant species were tested in different experiments and there were significant block (experiment) effects for some plant species, the analysis was done separately for each plant species.

For seed weight, the data were averaged and employed to determine the standard deviation of the mean from three replications.

2.3. Results

2.3.1. Pathogenicity of *T. basicola* isolates towards plants

Reaction of *T. basicola* isolates to plants

The degree of black root rot caused by *T. basicola* isolates was markedly different among the six isolates. The cotton, pansy and lupin isolates were highly pathogenic causing 64-80% necrotic lesions on the roots of cotton seedlings whereas the two lettuce and the carrot isolates were non-pathogenic on cotton seedlings. All *T. basicola* isolates except for the carrot isolate were highly pathogenic towards lupin seedlings causing 80-90% necrotic lesions (Figure 2.2).

The two lettuce isolates were highly virulent causing 69% necrotic lesions to lettuce seedlings, whereas the other isolates were weakly virulent or non-pathogenic to the lettuce seedlings (Figure 2.2). The carrot isolate was highly virulent on carrot roots causing 78% necrotic lesions, whereas the lupin and the two lettuce isolates were weakly virulent towards carrot. The cotton and pansy isolates were non-pathogenic to carrot seedlings. All the *T. basicola* isolates caused severe disease on pansy seedlings with 68-95% necrotic lesions. *T. thielavioides*, (carrot BT) was non-pathogenic to all the seedlings (Figure 2.2). All *T. basicola* isolates were weakly pathogenic or non-virulent to broccoli (Figure 2.2) or wheat seedlings (data not shown)

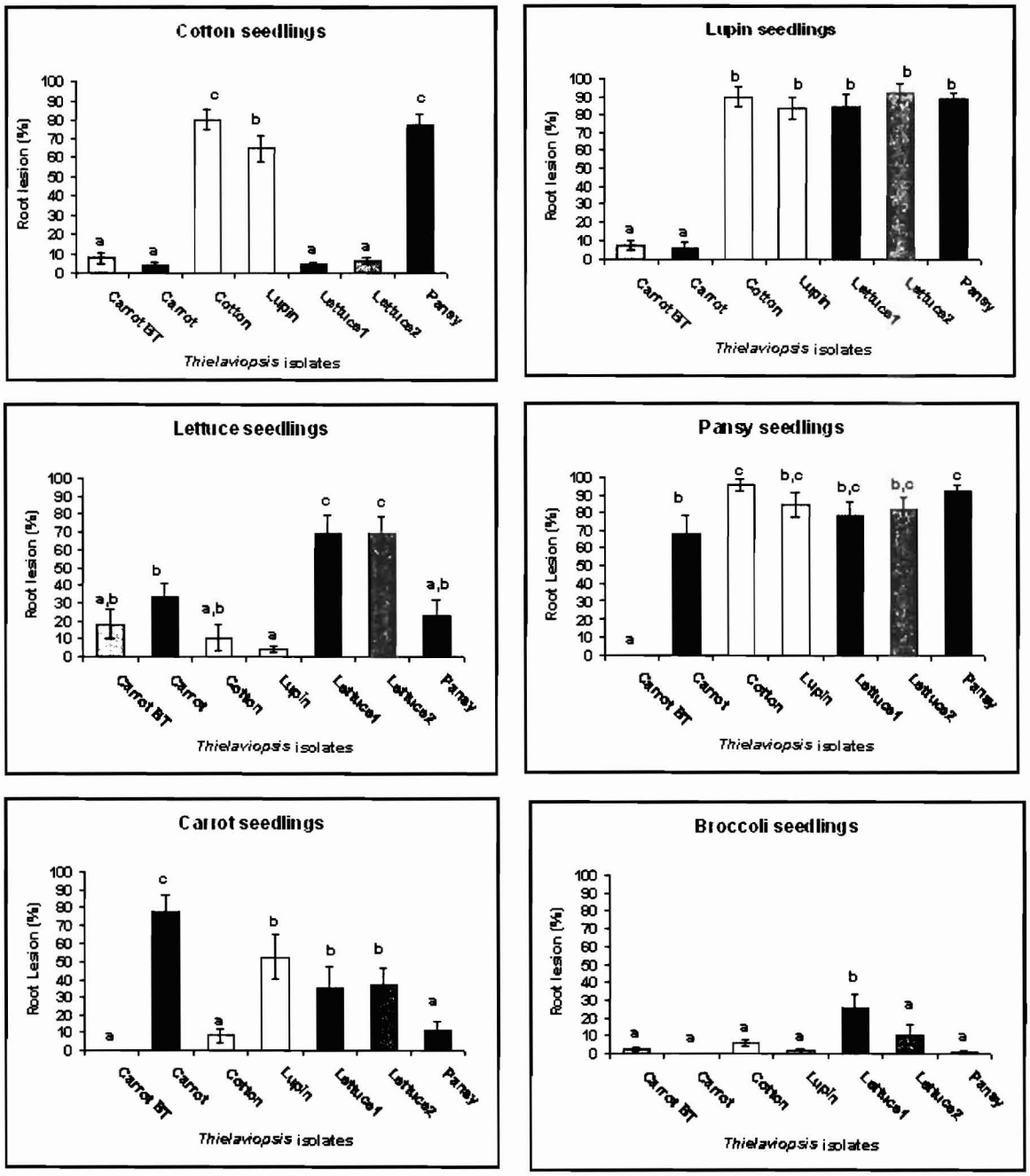

Figure 2.2. Assessment of pathogenicity of *Thielaviopsis* isolates on roots of seedlings

The Y-axis shows percentage of root lesions of each plant inoculated with 3.5×10^5 spores/mL recorded 7 days post inoculation. The X-axis represents the seven *Thielaviopsis* isolates used to inoculate root seedlings of each plant. Columns within a plant species labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Bars represent standard errors of the mean from 3 combined experiments.

Control seedlings not inoculated with endoconidia were free from lesions and the root system appeared healthier than inoculated roots (Figure 2.3 A&B).

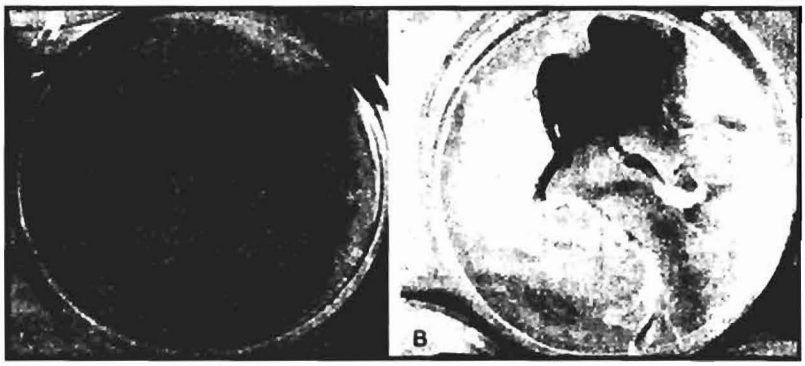

Figure 2.3. Pathogenicity tests using the dipping technique

(A) shows root lesions of 7 day old cotton seedlings inoculated with 3.5×10^5 endoconidia/mL of the cotton isolate (BRIP40192) on water agar (1.2%) plates. The length of root lesions was measured with a ruler as well as the total root length. Disease severity was expressed as the percentage of lesions out of the total root length. (B) shows 7 day old cotton seedlings not inoculated with endoconidia (free from lesions) on water agar (1.2%).

Reaction of plants to *T. basicola* isolates

The pathogenicity test results showed evidence that lupin, cotton, lettuce, pansy and carrot seedlings were susceptible to black root rot because several *T. basicola* isolates were capable of colonizing and invading their root system. The presence of necrotic lesions and protrusion of chlamydospores when observed under the stereomicroscope confirmed that these seedlings were hosts of *T. basicola* isolates. Therefore, I will refer to them as being susceptible host plants to most *T. basicola* isolates. Figure 2.4 shows roots of cotton and lupin seedlings infected with *T. basicola isolates*. At the concentration of spore inoculum used in this study, broccoli seedlings and wheat seedlings (data not shown) were weakly or not affected by all *T. basicola* isolates even though chlamydospores were seen on the broccoli and wheat roots when observed under the stereomicroscope. There was evidence that broccoli and wheat are also hosts to *T. basicola* but non-susceptible to the disease meaning that the isolates were able to invade the broccoli and wheat roots but were unable to cause necrosis, and therefore, they are considered as non-susceptible hosts. Rice

seedlings were not affected by any isolates and showed no symptoms of black root rot. They were free from any evidence of pathogen colonization when observed under the stereomicroscope and therefore represent a non-host of *T. basicola.*

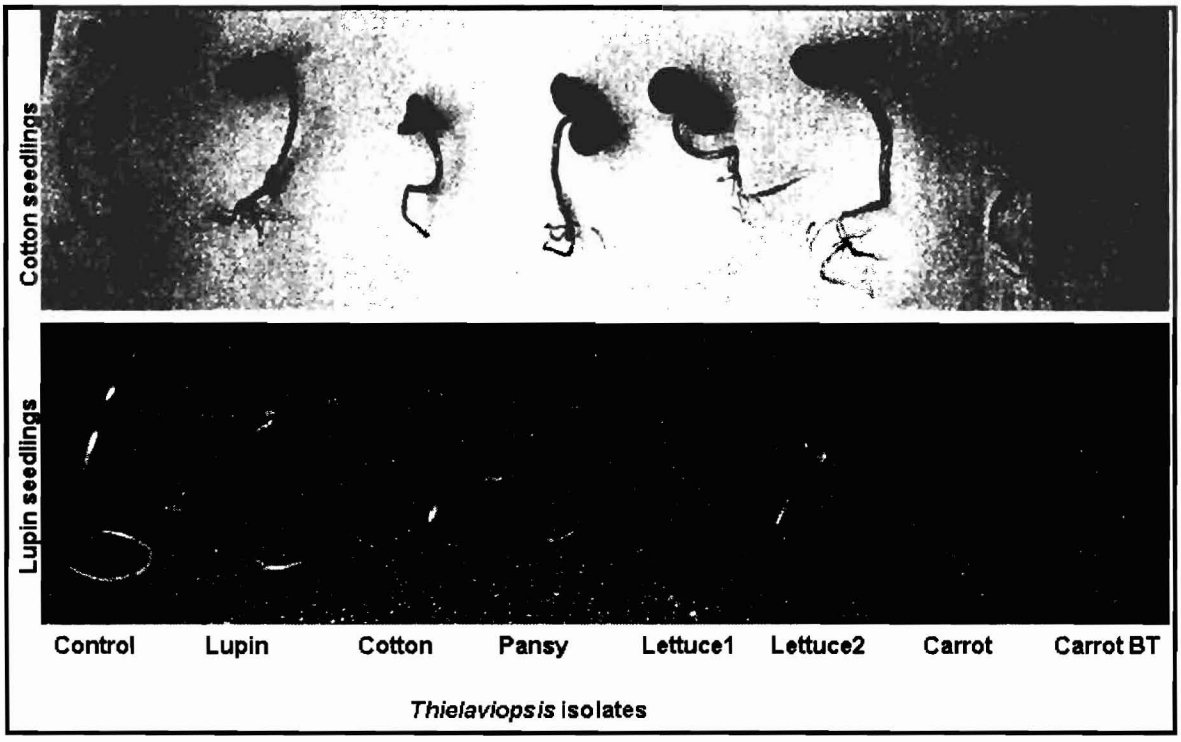

Figure 2.4. Representative roots of cotton and lupin seedlings infected with endoconidia of *Thielaviopsis* isolates showing different levels of black root rot.

2.3.2. Growth rates of *Thielaviopsis isolates* on ½ PDA (2.2% agar) and 1.2% water agar

Growth rates of *Thielaviopsis* isolates were studied on ½ PDA (2.2% agar) and water agar (1.2%) in order to account for natural differences in growth rate which are not related to the presence of a plant exudate or signal. Significant differences were observed in the growth rates between isolates on the two types of media tested (Figure 2.5). In PDA where plenty of nutrients were available, the highest growth rates were observed with carrot BT, cotton and lettuce1 isolate and the lowest growth rate were observed with the lupin and lettuce2 isolate. On 1.2% water agar, where the availability of nutrients was lower than in

PDA, carrot BT, pansy and the two lettuce isolates demonstrated high growth rates. The carrot isolate differed significantly from all the other isolates demonstrating the lowest growth rate.

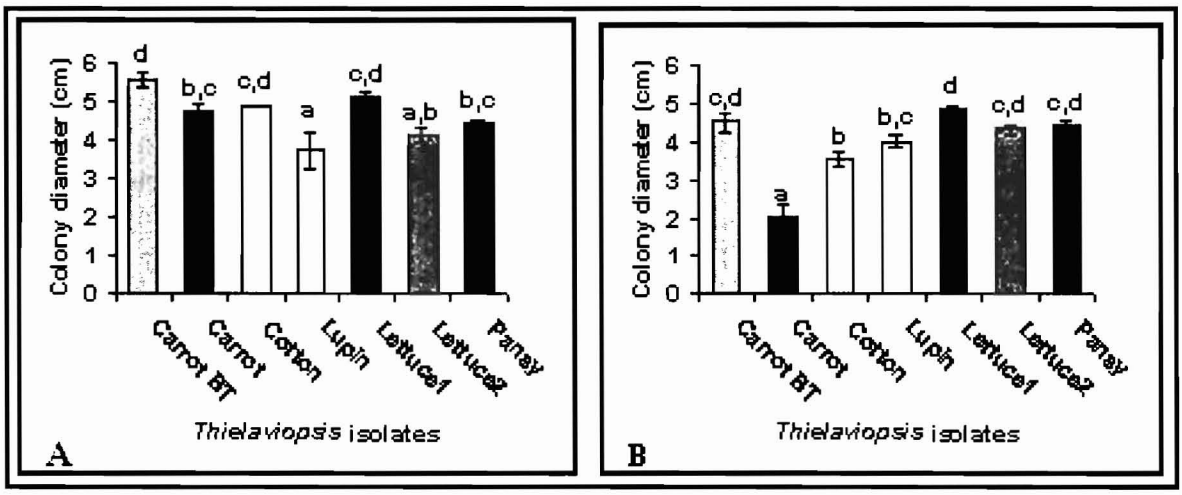

Figure 2.5. Assessment of growth rate of *Thielaviopsis* isolates

Colony diameter of *Thielaviopsis* isolates on ½ PDA (2.2% agar) (A) and 1.2% water agar (B) after 7 days of incubation at 25°C. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Bars represent standard errors of the mean from 3 combined replicates.

2.3.3. Directional growth of *Thielaviopsis* isolates towards plants

The directional growth data for each isolate was adjusted using relative growth rates for each isolate obtained on ½ PDA (2.2% agar) plates and not on 1.2% water agar plates because the growth in the presence of nutrients on ½ PDA (2.2% agar) was considered more relevant to growth in the presence of root exudates.

Reaction of *T. basicola* isolates to plants

All *T. basicola* isolates showed directional growth towards susceptible host plants (cotton, lupin or lettuce) with the strongest directional growth noted towards cotton and lupin seeds (Figure 2.6). Control treatments of endoconidial strips demonstrated no directional growth towards sterile strips containing 70 µL of de-ionised water.

Though all *T. basicola* isolates showed directional growth towards cotton seedlings, the cotton isolate (0.8 cm) demonstrated the strongest directional growth towards the cotton seedlings. The lupin isolate (0.57 cm) exhibited directional growth towards the lupin seedlings but was surpassed by the pansy isolate (1 cm), which exhibited the strongest directional growth towards lupin. The carrot BT isolate showed the weakest directional growth towards lupin seedlings. There was a significant difference in the response of the two lettuce isolates towards the lettuce seedlings. Lettuce1 (0.29 cm) showed stronger directional growth than lettuce2 (0.14 cm). The lupin isolate exhibited the weakest directional growth towards the lettuce seedlings. All *T. basicola* isolates showed directional growth towards the non-susceptible host plants broccoli and wheat, but did not show any directional growth with rice seeds (non-host) (data not shown). The cotton, lettuce1 and pansy isolates showed strong directional growth towards broccoli seedlings, whereas all the other isolates demonstrated weak directional growth (Figure 2.6). The cotton and lettuce1 isolates demonstrated strong directional growth towards wheat seedlings followed by the lupin and carrot isolate. The lettuce2 and pansy exhibited weak directional growth towards wheat seedlings. *T. thielavioides* (carrot BT) exhibited weak directional growth towards all seedlings except towards cotton seedlings where its directional growth was intermediate.

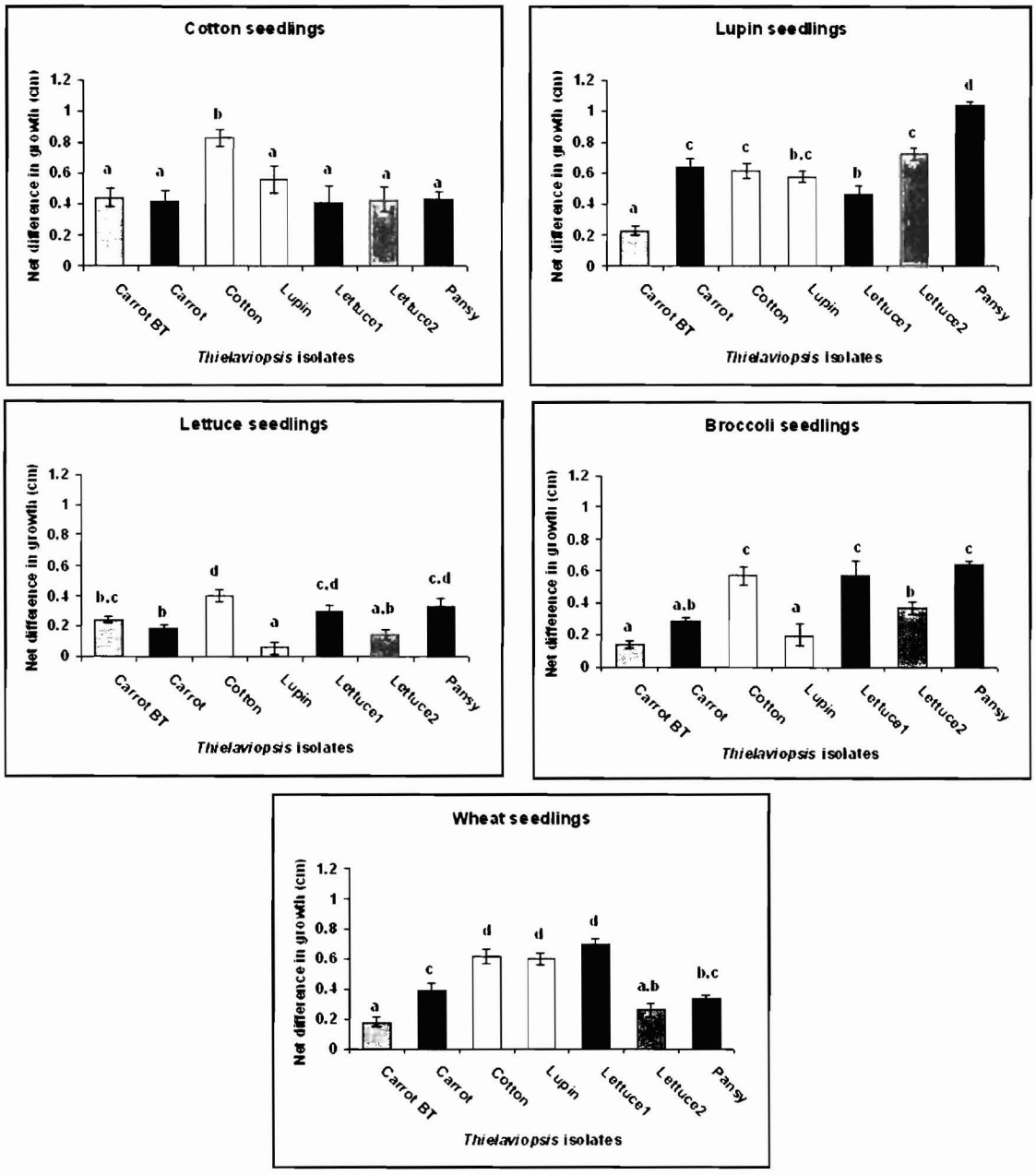

Figure 2.6. Assessment of directional growth efficiency of *Thielaviopsis* isolates towards seedlings

The Y-axis shows net differences in growth (in cm) towards and away from germinating seeds of each plant adjusted for relative growth rates of the isolates. Growth of mycelia was measured seven days post inoculation. The X-axis represents the seven *Thielaviopsis* isolates used to assess the directional growth efficiency towards germinating seedlings of each plant. Inoculum of 70 µL at a concentration of 3.5×10^5 endoconidia/mL was applied to filter paper strips and exposed to 4 seeds of each plant per plate. Columns within a plant species labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Bars represent standard errors of the mean from 3 combined experiments with 5 replicates in each experiment.

Chlamydospore formation was induced when *T. basicola* isolates were exposed to cotton, broccoli or wheat seedlings following 4-5 days incubation. Figure 2.7 below shows the cotton isolate demonstrating a significant strong hyphal directional growth and chlamydospore formation towards cotton seedlings. Chlamydospore production was absent or sparse when *T. basicola* isolates were exposed to lupin, lettuce or rice seeds. *T. thielaviodes* (carrot BT) did not demonstrate chlamydospores when exposed to any of the seedlings. All control treatments lacked chlamydospores.

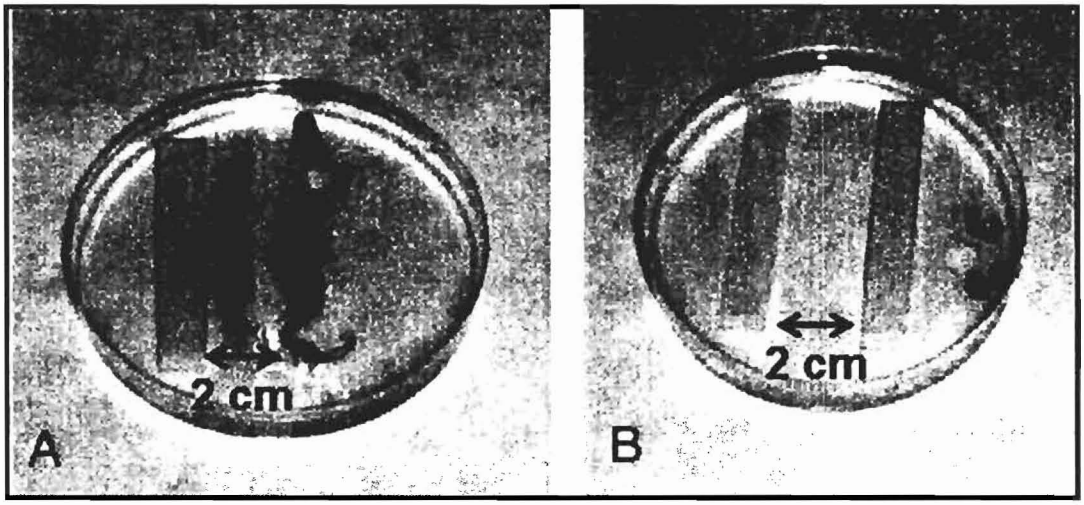

Figure 2.7. Growth of *Thielaviopsis basicola* (cotton isolate) towards cotton seedlings

(A). shows growth of dark brown mycelia of the cotton isolate of *T. basicola* growing away from the strip (that was inoculated with 70 µl of 3.5 × 10^5 endoconidia/mL) towards cotton seedlings (*G. hirsutum*) after 7 days incubation. The mycelia contain numerous chlamydospores when observed under the stereomicroscope. The seedlings were placed at a distance of 2 cm from the endoconidia strip. (B). shows control plate containing a strip inoculated with 70 µL of 3.5 × 10^5 endoconidia/mL of the cotton isolate (left strip) exposed to a second strip containing 70 µl of de-ionised water (right strip) instead of seedlings. The distance between the two strips is 2 cm. Growth of the cotton isolate is not observed.

2.3.4. Host preference tests

The directional growth assays showed evidence that all *Thielaviopsis* isolates grew towards stimulants released from roots of all seeds except rice (non-host) (Figure 2.6). However, it was not known whether an isolate grows preferentially towards the host from which it was isolated from, when other seedlings are present. Therefore, preference tests were conducted

where each *T. basicola* isolate was exposed to the same or two types of seeds simultaneously using either the number of seeds or the weight of seeds as a variant. Control treatments demonstrated either no growth or equal growth on both sides of sterile strips containing 70 μL de-ionised water.

2.3.4.1. Determining preferential growth of *T. basicola* isolates towards hosts using number of seeds

Cotton isolate

When the cotton isolate was challenged with 6 cotton seeds versus 12 seeds of each of lupin, lettuce, broccoli, rice or wheat, there was more mycelial growth towards the cotton seedlings (Table 2.5). This is consistent with the data presented in Figure 2.6, where the cotton isolate demonstrated stronger directional growth towards cotton seedlings than towards other seedlings. When the cotton isolate was challenged with 6 cotton seeds versus 6 cotton seeds growth was similar on both sides. When the cotton isolate was tested with 6 cotton seeds versus 4 cotton seeds or 2 cotton seeds, the cotton isolate showed more growth towards 6 seeds (Table 2.5).

Table 2.5. Preferential growth of cotton isolate towards cotton seeds or seeds of other plants

Other plants	Cotton (6 seeds)
Cotton (6 seeds)	=
Cotton (4 seeds)	+
Cotton (2 seeds)	+
Lupin (12 seeds)	+
Lettuce (12 seeds)	+
Broccoli (12 seeds)	+
Wheat (12 seeds)	+
Rice (12 seeds)	+

(=): same rate of growth or no preference
(+): more growth towards cotton seeds (6 seeds) than towards other plants

Lupin isolate

When the lupin isolate was challenged with six lupin seedlings versus 12 seedlings of each of lettuce, broccoli, or rice per plate, the lupin isolate showed more growth towards the lupin seedlings (Table 2.6). These results confirm the data presented in Figure 2.6 (lupin, lettuce and broccoli seedlings) where directional growth of the lupin isolate was greater towards lupin seedlings (0.58 cm) than towards lettuce seedlings (0.05 cm) or broccoli seedlings (0.19 cm) when tested individually.

When the lupin isolate was challenged with six lupin seeds versus 12 wheat seedlings per plate, the lupin seedlings appeared to be more stimulatory than the wheat seedlings (Table 2.6). However, the data from the previous experiment (Figure 2.6) demonstrated that the four lupin seedlings (0.57 cm) and four wheat seedlings (0.59 cm) showed similar stimulation by the lupin isolate when tested individually. When the lupin isolate was challenged with 6 lupin seeds versus 12 cotton seedlings, the lupin isolate demonstrated more growth towards the cotton seedlings than towards the lupin seedlings (Table 2.6). This was also evidenced whereby four cotton seedlings (0.55 cm) and four lupin seedlings (0.57 cm) demonstrated similar stimulation of the lupin isolate when tested individually (Figure 2.6). When the lupin isolate was challenged with 6 lupin seeds versus 2, 4 or 6 lupin seeds, directional growth was similar on both sides (Table 2.6).

Table 2.6. Preferential growth of lupin isolate towards lupin seeds or seeds of other plants

Other plants	Lupin (6 seeds)
Lupin (6 seeds)	=
Lupin (4 seeds)	=
Lupin (2 seeds)	=
Cotton (12 seeds)	-
Lettuce (12 seeds)	+
Broccoli (12 seeds)	+
Wheat (12 seeds)	+
Rice (12 seeds)	+

(=): same rate of growth or no preference
(-): more growth towards other plants than towards lupin seeds (6 seeds)
(+):more growth towards lupin (6 seeds) than towards other plants

Lettuce1 isolate

The lettuce1 isolate showed more growth towards lettuce seedlings (respective host) when challenged with 6 lettuce seeds versus 12 seeds of rice (Table 2.7). When the lettuce1 isolate was challenged with 6 lettuce seeds versus 12 cotton, broccoli or wheat seedlings, the lettuce1 isolate demonstrated more growth towards cotton, broccoli or wheat seedlings than towards lettuce seedlings. This data is consistent with the data obtained in Figure 2.6 where equal numbers of seeds were used. The lettuce1 isolate demonstrated a strong directional growth towards cotton (0.41 cm), broccoli (0.57 cm) and wheat (0.69 cm) seedlings than towards lettuce seedlings (0.29 cm) when tested individually.

A similar pattern was observed in Figure 2.6 demonstrating that lupin seedlings (0.46 cm) were more stimulatory to the lettuce1 isolate than the lettuce seedlings (0.29 cm) when tested individually using an equal number of seeds. However, the lettuce1 isolate showed more growth towards the lettuce seedlings (6 seedlings in number) when challenged versus 12 seedlings of lupin (Table 2.7). When the lettuce1 isolate was challenged with 6 lettuce seeds against 2, 4 or 6 lettuce seeds, directional growth was similar on both sides.

Table 2.7. Preferential growth of the lettuce1 isolate towards lettuce seeds or seeds of other plants

Other plants	Lettuce (6 seeds)
Lettuce (6 seeds)	=
Lettuce (4 seeds)	=
Lettuce (2 seeds)	=
Cotton (12 seeds)	-
Lupin (12 seeds)	+
Broccoli (12 seeds)	-
Wheat (12 seeds)	-
Rice (12 seeds)	+

(=): same rate of growth or no preference
(-): more growth towards other plants than towards lettuce (6 seeds)
(+):more growth towards lettuce (6 seeds) than towards other plants

2.3.4.2. Determining preferential growth of *T. basicola* isolates towards hosts using equal weight of seeds

In order to determine the effect of stimulation of equal weight of seeds exposed to *T. basicola* isolates, additional preference tests were conducted. Previous data revealed that the lupin and lettuce1 isolate showed lower directional growth towards lupin and lettuce seedlings, respectively, than towards cotton seedlings when different numbers of seeds were used (Table 2.6 and Table 2.7). When the lupin and lettuce1 isolate were exposed to equal weight of two types of seeds simultaneously, lupin versus cotton seedlings and lettuce versus cotton seedlings respectively, it was found that the lupin and the lettuce1 isolates still showed greater growth towards the cotton seedlings (Table 2.8). Furthermore, when the weight of the lupin seeds and the lettuce seeds were increased compared to the weight of the cotton seeds, the lupin and lettuce1 isolate still showed greater growth towards the cotton seedlings.

Previous data showed that the lettuce1 isolate demonstrated lower directional growth towards lettuce seedlings than towards broccoli and wheat seedlings when number of seeds was used (Table 2.7). When the equal weight of lettuce versus broccoli seeds and lettuce versus wheat seeds were used, the lettuce1 isolate still showed preference in its directionality towards broccoli and wheat seeds (Table 2.8).

Table 2.8. Preferential growth of the lupin and lettuce1 isolates towards seeds of equal weight.

Thielaviopsis basicola isolate	Type of seed	Approximate weight of seeds (g)	Preferential growth towards
Lupin	Lupin	0.55 ± 0.06	Cotton seedlings
	Cotton	0.55 ± 0	
Lettuce1	Lettuce	0.18 ± 0.04	Cotton seedlings
	Cotton	0.18 ± 0.08	
Lupin	Lupin	1.12 ± 0.04	Cotton seedlings
	Cotton	0.55 ± 0	
Lettuce1	Lettuce	0.25 ± 0.04	Cotton seedlings
	Cotton	0.18 ± 0.08	
Lettuce1	Lettuce	0.02 ± 0.01	Broccoli seedlings
	Broccoli	0.02 ± 0.05	
Lettuce1	Lettuce	0.14 ± 0.02	Wheat seedlings
	Wheat	0.14 ± 0.05	

± :- represents standard deviation of the mean from three replications

2.4. Discussion

T. basicola is a widespread pathogen that infects the epidermal and cortical tissues of roots and hypocotyls of a diverse range of plant species (Christou, 1962). In view of the wide host range of *T. basicola*, it is possible that isolates overlap in their pathogenicity on some hosts but are specific to others (Keller and Shanks, 1955). In pathogenicity tests, the cotton isolate demonstrated a low level of host specificity as it was found to be highly virulent on cotton seedlings, however, its severity on lupin and pansy seedlings was greater. The two lettuce isolates were also found to demonstrate a low level of host specificity. Although they were more virulent on lettuce seedlings than any of the other isolates, they were more severely pathogenic to lupin and pansy seedlings than to lettuce. The failure of the two lettuce isolates to cause black root rot in cotton seedlings is consistent with the results of O'Brien and Davis (1994) who inoculated cotton roots by root dipping in a conidial suspension of 1×10^6/mL. The lupin and pansy seedlings appeared to be the most susceptible hosts to most of the *T. basicola* isolates.

Inoculum concentration of endoconidia is important to show differences in the specificity of various isolates (Tabachnik *et al.* 1979). Tabachnik *et al.* (1979) obtained the best discrimination between isolates at an inoculum concentration of 10^4 endoconidia per gram of soil, which caused lesions on all susceptible hosts. It is possible that the *T. basicola* isolates used at an inoculm concentration of 3.5×10^5 endoconidia/mL in the current study did not show differences in their virulence to lupin and pansy seedlings, however, they gave a wide range of disease severities on most hosts. Rice apparently was not affected by *T. basicola* isolates at the inoculum concentration used and served as a non-host.

During pathogenesis by *T. basicola*, the production of chlamydospores begins several days after infection, which corresponds with the death of host cells (necrosis) (Hood and Shew, 1997b). Hood and Shew (1997a) also demonstrated that chlamydospore production may

occur in the absence of additional nutrient acquisition *in vitro*. The current study demonstrated that although necrosis was not evident in broccoli and wheat seedlings, production of chlamydospores was apparent on broccoli and wheat seedlings when observed under the stereomicroscope, suggesting that the absence of necrosis could indicate that broccoli and wheat are hosts but not susceptible to disease.

Among all the *Thielaviopsis* isolates, carrot BT known as *Thielaviopsis thielavioides* was the weakest pathogen causing little or no disease on any of the seedlings and served as a negative control.

Becard and Piche (1989) reported that hyphae of mycorrhizal fungi elongate 20 times more slowly in the absence of host roots than in their presence. The directional attraction may not be a general phenomenon, but may be more characteristic of the specific host tested (Vierheilig *et al.*, 1998). The present study used endoconidia and Noble water agar to study directional growth of *T. basicola* isolates. Noble agar which lacks nutrients, a condition analogous to nutrient stress would lead to the induction of chlamydospores as observed by Hood and Shew (1997a) when they replaced the nutrient solution with sterile de-ionised water in the endoconidial suspension (Hood and Shew, 1997a). The availability of high concentration of nutrients results in the lack of chlamydospore production but as nutrients become depleted, chlamydospores are formed in response to a decrease in the level of available nutrients (Hood and Shew, 1997a). The chlamydospore induction method developed by Hood and Shew (1997a) was used as an informative base in the present study for directional growth. The induction of chlamydospores in Noble agar in the presence of germinating seedlings would indicate that the germinating seedlings are secreting nutrients into the agar and the spores are utilizing these nutrients for their germination and hyphal growth. But as these nutrients continue to be utilised by the growing fungi faster than more nutrients are being secreted by the roots, leading to the decrease of available nutrients, thus

triggering the formation of chlamydospores. Chlamydospores are considered to be the primary overwintering propagules of *T. basicola* (Tsao and Bricker, 1966). Their production has been reported to serve as a measure of pathogen fitness (Tosi and Zazzerini, 1991). In directional growth tests, a dense mycelial mat constituting of a substantial number of chlamydospores was more evident at day 4-5 when *T. basicola* isolates were exposed to cotton, broccoli and wheat seedlings. It is possible that the roots of these seeds secrete a substantial amount of exudates and that the production of chlamydospores corresponded to the depletion of root exudates in the Noble agar.

In cases where chlamydospores were not significantly evident when *T. basicola* isolates were exposed to lupin, lettuce and rice seeds, it could be that the roots of these seeds do not secrete enough exudates to induce fungal growth and the production of chlamydospores. The mycelia were not dense and were similar to the visual appearance of mycelia inoculated in 1.2% water agar plates. Hood and Shew (1997) also observed that the length of the hyphae and degree of branching of chlamydospores decreased under nutrient stress.

All isolates showed a directional growth response towards cotton, lupin, lettuce, broccoli and wheat indicating a low level of host specificity. It is possible that the seedlings did not show differences in the specificity of the various isolates used at an inoculum concentration of 3.5×10^5 endoconidia/mL in the current study.

In host preference tests using the number or weight of seeds, it was found that in some cases, an isolate showed stronger directional growth towards its respective host than any other susceptible host, such as the cotton isolate on cotton seedlings. In other cases, it was found that an isolate showed stronger directional growth towards a different susceptible host than its respective host, such as the lettuce1 isolate with cotton or lupin seedlings. It was also found that isolates could demonstrate stronger directional growth towards non-susceptible hosts than their respective hosts, such as the lettuce1 isolate on broccoli and

wheat seedlings. These results indicate that the quantity of root exudates of seedlings may play a role in influencing germination and directionality rather than a host plant secreting specific substances that promote more growth of a particular isolate towards its respective host. However, further analysis in quantifying the amount of exudates released from seeds would require confirming this preliminary observation. Root exudates of both host and non-host plants have been previously reported to non-specifically trigger germination of spores of fungal root pathogens (Lockwood, 1977; Deacon, 2006). This has been exploited in disease management especially in traditional crop-rotation systems where the spores of pathogenic fungi can be induced to germinate but then die because they cannot infect a plant, a phenomenon known as germination-lysis (Lockwood, 1977; Deacon, 2006). It is possible that broccoli and wheat could be exploited in the management of black root rot.

In conclusion, the pathogenicity and directional growth results obtained in this study demonstrated three phenomenona whereby an isolate showed 1) both strong directional growth and virulence towards hosts, such as cotton and lettuce1 isolates on cotton and lettuce seedlings, respectively; 2) strong directional growth but no pathogenicity, such as the two lettuce isolates and the two carrot isolates on cotton seeds, the two carrot isolates on lupin seedlings and the cotton isolate on lettuce seedlings; and 3) weak directional growth but high virulence, such as the lettuce2 isolate on lettuce seedlings. These data suggest that isolates that exhibit a directional growth response towards a particular susceptible plant do not necessarily cause disease and isolates that severely infect susceptible plants can demonstrate a weak directional growth response. The ability of the lettuce2 isolate to cause heavy infection on the lettuce seedlings in response to a weak directional growth response could be explained from the pathogenicity tests where lettuce seedlings were dipped in an endoconidial suspension thus allowing the endoconidia to attach directly to the roots rather than the endoconidia having to grow towards the roots.

These results also indicate that strains isolated from particular hosts may exhibit strong growth direction and/or pathogenicity towards other hosts. For example, the pansy isolate demonstrated strong directional growth and virulence towards lupin seeds as well as pansy seeds.

This study offers some evidence that nutrients released by roots of hosts and non-hosts are responsible for *T. basicola* hyphal directional growth. However, there is little evidence to suggest that a host-specific stimulus causes hyphal growth direction. The fact that root exudates stimulate hyphal growth direction of a pathogen need not necessarily be construed as enhancing pathogenesis, since successful parasitism depends on the completion of a sequence of steps in pathogenesis. The constituents of host exudates and the concentration required to influence spore germination and hyphal growth direction may or may not be optimal for production of toxins, or production of other metabolites which contribute to the pathogenicity of an organism at the given experimental conditions. Therefore, the conclusions drawn from this *in vitro* study do not necessarily reflect what happens in the field. When relating disease susceptibility of a plant, caution should be exercised. The composition of root exudates has been reported to vary with conditions of the test and during the life of the plant (Schroth and Hildebrand, 1964). For example, a certain temperature and humidity may favour the exudation of amino acids from one plant species, yet with another plant species, the same conditions might cause an increase in the exudation of carbohydrates. Therefore, such evidence would be more convincing if root exudates were collected and tested against a pathogen under a range of conditions. Also when establishing a relationship between host root exudation and its pathogen in causing disease, more information is needed on the composition and quantification of root exudates to answer the question: what are the substances exuded and the quantity required to stimulate germination and cause disease?

Chapter 3 . PEG-mediated transformation of *T. basicola*

3.1. Introduction

Fungal plant pathogens employ diverse strategies to infect their hosts. Mechanisms underlying these strategies are being revealed by the application of molecular genetic techniques including insertional mutagenesis. Random insertional mutagenesis is a powerful strategy to identify fungal genes involved in pathogenicity. This approach does not require *a priori* knowledge of genes involved in fungal pathogenicity. Random insertional mutagenesis causes disruption of a pathogenicity gene resulting in significant or complete loss of host disease symptoms when mutants are inoculated onto hosts that are susceptible to the wild-type pathogen.

The infection process of *T. basicola* is well characterised. However, knowledge of the mechanisms used by *T. basicola* to parasitise *Gossypium hirsutum* (cotton) remains inadequate. This is due, in part, to the lack of molecular tools available to characterise pathogenicity and virulence factors of this fungus and an incomplete understanding of specific host/pathogen interactions. The development of an efficient genetic transformation system has the potential to advance studies of the pathogen and may ultimately lead to a broader range of control strategies. A transformation system can be used for random insertional mutagenesis of *T. basicola* which potentially leads to the identification of genes and pathways responsible for *T. basicola* pathogenicity and their control. Several methods for transformation of filamentous fungi have been reviewed (Lemke, 1995; Mullins and Kang, 2001; Ruiz-Diez, 2002; Olmedo-Monfil *et al.*, 2004; Michielse *et al.*, 2005). The methods include the following:

1. PEG-mediated transformation whereby protoplasts are incubated with DNA and calcium chloride prior to the addition of PEG (polyethylene glycol) as a fusionogenic medium. PEG causes the protoplasts to clump together, which facilitates the trapping of DNA. The optimal conditions for protoplast production and regeneration of cell walls from the protoplasts need to be established for individual fungi. The key factors include: the type and concentration of lytic enzymes used, enzymatic digestion time, concentration of PEG, age and concentration of protoplasts, and type and concentration of osmotic stabiliser to stabilise the protoplasts and to regenerate the cell walls (Case *et al.*, 1979; Fincham, 1989; Kuspa and Loomis, 1992; Bowyer, 2001).
2. Restriction enzyme mediated integration (REMI) is similar to PEG-mediated transformation except that the circular transforming plasmid DNA is linearized at a single site. The same restriction enzyme is added in the transformation mix to facilitate integration of DNA through the production of compatible cohesive ends (Schiestl and Petes, 1991).
3. Electroporation, where high voltage electric pulses delivered by a Gene Pulser apparatus are applied to pre-treated conidia with a cell wall weakening agent or directly to protoplasts, resulting in structural rearrangements of the cell membrane, creating temporary pores for the uptake of DNA (Richey *et al.*, 1989; Chakraborty *et al.*, 1991).
4. Lithium acetate treatment, a procedure that avoids protoplast formation and involves exposing germinating endoconidia to the transforming DNA in the presence of 0.1 mol l^{-1} lithium acetate (Dhawale *et al.*, 1984).
5. Biolistic transformation, whereby tungsten particles coated with DNA are accelerated at high velocity directly into fungal spores or hyphae (Klein *et al.*, 1987).

6. *Agrobacterium tumefaciens*-mediated transformation (ATMT), a procedure which alleviates protoplast production, as spores, hyphae, or blocks of mycelia can be transformed directly (de Groot *et al.*, 1998).

The majority of plasmid DNA used in transformation of filamentous fungi integrate into the fungal chromosome. The plasmid DNA used can be circular or linear. In ATMT, a single stranded linear segment of transfer-DNA (T-DNA) from *A. tumefaciens*, is transferred and integrated into a fungal genome depending on the induction of a set of virulence genes that occur on the *vir* plasmid of the binary vector system (Lemke, 1995; Olmedo-Monfil *et al.*, 2004; Michielse *et al.*, 2005).

PEG- mediated transformation of protoplasts is the most common approach that has been used for transformation of many filamentous fungi (Riach and Kinghorn, 1996). To date, there has been no report on the genetic transformation of *T. basicola*. In this chapter, I report for the first time, transformation of the filamentous fungus, *T. basicola*, using the PEG/$CaCl_2$ method. The plasmid DNA used for random insertional mutagenesis, pGpdGFP (Sexton and Howlett, 2001), contains antibiotic resistance marker genes for *Escherichia coli* and fungi. Resistance in *T. basicola* was conferred by a dominant selectable marker, the *E. coli* hygromycin B phosphotransferase (*hph*) gene. Integration of the transforming DNA into the *T. basicola* genome was confirmed by Southern hybridisation analysis. The putative transformants were characterised for mitotic stability of the integrated plasmid DNA.

3.2. Materials and Methods

3.2.1. Fungal strain and maintainance

Transformation experiments were all carried out using *T. basicola* strain BRIP40192, isolated from cotton (*G. hirsutum*), which was kindly provided by Jan Dean, Department of Primary Industries, Queensland Government, Australia. Stock cultures of *T. basicola* were preserved as mycelial agar blocks in 10% glycerol at -70°C or maintained in sterile distilled water at room temperature. Working cultures were grown at 25°C on ½ PDA (2.2% agar) (section 2.2.2.1) and stored at 4°C until needed.

3.2.2. Plasmid and bacterial strain

A circular plasmid pGpdGFP (6.93 kb) was used for PEG-mediated transformation of *T. basicola* to generate hygromycin resistant fungal transformants. This plasmid was also used to transform *Leptosphaeria maculans* by the REMI method (Sexton and Howlett, 2001). Plasmid pGpdGFP was kindly provided by Barbara Howlett, School of Botany, University of Melbourne, Australia (Figure 3.1). The plasmid was engineered by Dr. A. Andrianopolous, Genetics Department, the University of Melbourne, Australia (Sexton and Howlett, 2001). It contains the hygromycin phosphotransferase gene (*hph*) from *E. coli*. Expression of *hph* is controlled by the *Aspergillus nidulans trpC* gene promoter (P*trpC*) and *trpC* gene transcription-termination (T*trpC*) signals. Plasmid pGpdGFP was maintained in *E. coli* cells (DH5α strain) and stocks were kept in glycerol stocks at –70°C.

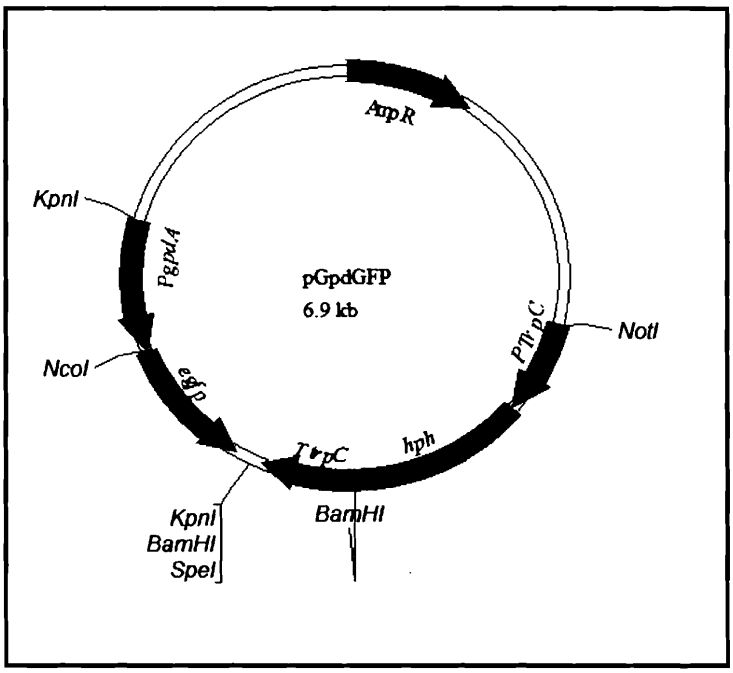

Figure 3.1. Diagram of plasmid pGpdGFP (6.93 kb).

The plasmid DNA contains an ampicillin resistance (*Amp*R) gene, a hygromycin B resistance (*hph*) gene from *E. coli* controlled by *A. nidulans trp*C promoter (*Ptrp*C) and *A. nidulans trpC* terminator (T*trpC*). It also contains a gene for green fluorescent protein (egfp) controlled by *A. nidulans* glyceraldehyde dehydrogenase phosphate promoter (P*gpd*A) *and A. nidulans trpC* terminator (*TtrpC*).

3.2.3. *E. coli* DH5α transformation.

A number of bacterial transformations of *E. coli* DH5α cells, made competent with 0.1 M $CaCl_2$ treatment, were performed in order to store plasmid DNA which was used in this study. A transformation entailed incubation on ice of 100 µL of DH5α competent cells with the plasmid pGpdGFP (10 ng) for 30 min, followed by heat shock at 42°C for exactly 1 min and then the addition of 0.5 mL 2× Yeast Tryptone (YT) broth (section 3.2.6.5). The mixture was allowed to incubate at 37°C for 1 h with shaking at 200 rpm, before an aliquot of 100 µL was taken and plated on 2× YT agar (Sambrook *et al.*, 1989). The solid medium contained 100 µg/mL ampicillin and 1.5% (w/v) agar. The plates were then incubated overnight at 37°C. A negative control (which did not contain plasmid DNA) was performed.

3.2.4. Isolation of plasmid DNA

Plasmid DNA was propagated in *E. coli* strain DH5α, and purified using the QIAprep spin miniprep kit (QIAGEN) and midiprep kit (QIAGEN) according to the manufacturer's instructions and stored at -20°C in 10 mM Tris-HCl pH 8.0.

3.2.5. Materials used: chemicals, solutions and reagents

Table 3.1 lists the composition of all the solutions, buffers and reagents used in this chapter.

Table 3.1. List of solutions, buffers and reagents.

Material	Composition
Trapping buffer	0.6 M sorbitol, 10 mM Tris-HCl pH 7.0
1× STC	1.2 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$ pH 7.5
2× STC	2.4 M sorbitol, 20 mM Tris-HCL, 20 mM $CaCl_2$ pH 7.5
Tween solution	Tween 80 (0.1%) in distilled water
Osmotic medium	1.2 $MgSO_4 \cdot 7H_2O$, 10 mM sodium orthophosphate buffered at pH 5.8 with 0.2 M Na_2HPO_4 (sterilized by filtration)
PEG solution	60% (v/v) PEG 4000, 10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$
Gel loading dye	0.25% bromophenol blue, 0.25% xylene blue, 15% Ficoll (type 400) in H_2O
Ethidium bromide solution	10 mg/mL ethidium bromide
Hygromycin B (Sigma)	25 mg/mL stock solution in de-ionised water sterilised by filtration through a 0.2 μm cellulose acetate membrane.
Glucanex	(Lysing enzyme from *Trichoderma harzianum* Lot No. 102K1416 Sigma) 5 mg/mL in osmotic medium
1× TE buffer pH 8.0	10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0
1× TEAC buffer ph 7.6	40 mM Tris, 1 mM EDTA, 20 mM glacial acetic acid
Genomic DNA extraction buffer	100 mM Tris HCl pH 8.0, 10 mM EDTA, 2% SDS

3.2.6. Growth media and culture conditions

3.2.6.1. Potato dextrose broth (PDB)

Potato dextrose broth used for the germination of endoconidia contained the filtrate of 220 g of boiled potatoes and 20 g of D-glucose (dextrose) made up to 1 liter with distilled water.

3.2.6.2. ½ PDA media (1.2% agar)

½ PDA media (1.2% agar) used for plating the regenerated fungal protoplasts contained 1.95% (w/v) PDA medium and 0.45% (w/v) agar (Oxoid Ltd., Hampshire, England) and varying concentrations of hygromycin B (6, 25, 50 and 100 µg/mL) or without hygromycin B.

3.2.6.3. Regeneration solid media

The regeneration medium used for regeneration of fungal protoplasts contained 1.0 M or 1.2 M sucrose and ½ PDA (1% agar). ½ PDA (1% agar) contained 1.95% (w/v) PDA medium and 0.25% (w/v) agar. Selective regeneration solid medium was supplemented with varying final concentrations of hygromycin B (6, 25, 50 and 100 µg/mL) to select for transformants containing pGpdGFP.

3.2.6.4. Regeneration broth

Regeneration broth used for the regeneration of fungal protoplasts contained PDB supplemented with varying concentrations of sucrose (0.2 M, 0.5 M or 1.0 M).

3.2.6.5. 2× Yeast Tryptone growth broth or media, pH 7.0

2 × YT medium contained 1.6% (w/v) Tryptone, 1.0% (w/v) Yeast extract, and 0.5% (w/v) NaCl. For solid media 1.5% (w/v) agar was added (Sambrook *et al.*, 1989).

3.2.7. Protocol development for transformation of *T. basicola*

3.2.7.1. Preparation of fungal protoplasts

Germination of endoconidia

T. basicola cultures were grown on ½ PDA (2.2% agar) (90 × 14 mm, vented) plates for 3 days at 25°C and then transferred into 10 mL Tween 80 (0.1%). The mycelial suspension was vortexed to release endoconidia. Endoconidia were separated from mycelia and chlamydospores by filtering the suspension through one layer of sterile Miracloth (Calbiochem) (sterilized overnight by soaking in 70% ethanol) and collected in a 1 liter flask containing 200 mL PDB. The endoconidia were incubated at 25°C with 120 rpm agitation and harvested at differing times between 0 and 24 h and observed and photographed under the light microscope to determine the initiation of germination of endoconidia. Germinating endoconidia were then harvested by centrifugation for 5 min at 2500 × *g* at 4°C and washed 3 times with 15 mL ice-cold sterile 0.6 M $MgSO_4$ and centrifuged again for 5 min at 2500 × *g* at 4°C.

Lysis of the cell wall

After centrifugation, the supernatant was discarded and the wet weight of the germinating endoconidial pellet was recorded. According to the wet weight of the endoconidial pellet, the pellet was resuspended in 5, 10 or 15 mL of ice cold osmotic medium containing various concentrations (5, 10, 25 or 50 mg/mL) of Glucanex, and incubated for 2 h at 30°C with shaking at 120 rpm.

Separation of protoplasts from cell debris

Protoplasts were separated from cell debris using a modified procedure of Tilburn *et al.* (1983) as follows: An equal volume of the protoplast suspension was transferred to two sterile Corex tubes and each tube was overlayed with 5 mL of trapping buffer (Table 3.1).

The tubes were centrifuged at 4000 × *g* at 4°C for 20 min and protoplasts that were banded at the interface of the two layers were withdrawn using a sterile Pasteur pipette. Protoplasts were then resuspended in ice cold 10 mL of 1× STC (Table 3.1), pelleted at 5000 × *g* for 5 min at 4°C, washed two times with ice cold 10 mL of 1× STC. The washing step was repeated again as washing protoplasts with a suitable buffer prior to transformation is usually sufficient to remove contaminating nucleases. The protoplasts were finally counted using a haemocytometer chamber and adjusted as aliquots of 2×10^6 protoplasts in ice cold 150 µL 1× STC.

Optimizing protoplast regeneration media and determining true protoplasts

Aliquots of protoplasts were diluted 1:1000 with ice cold 1× STC or sterile distilled water and plated on non-selective regeneration media containing 1 M or 1.2 M sucrose. As distilled water causes true protoplasts to burst, no colonies were expected on regeneration media containing protoplasts resuspended in distilled water. Colonies that grew were transferred to ½ PDA (1.2% agar) plates lacking osmotic stabilizers and incubated at 25°C for 7 days.

Optimising protoplast regeneration broth and effect of PEG

To determine the optimal conditions and the effect of PEG on the regeneration of protoplasts, aliquots of protoplasts in 150 µL ice cold 1× STC were adjusted to 200 µL with ice cold 1× STC. Protoplasts were mixed with 100 µl of PEG solution or 100 µl of ice cold 1× STC and incubated on ice for 5 min. The treatments were then incubated on ice for 20 min. PEG solution (1 mL) was added to the PEG treatments and gently mixed. Treatments that did not contain PEG were gently mixed with ice cold 1 mL 1× STC. All tubes were incubated at room temperature for 15 min. Protoplasts were then pelleted at 20,000 × *g* (14, 000 rpm) for 2 min and washed with ice cold 1 mL 1× STC, collected by centrifugation at 20,000 × *g* (14,000 rpm), 2 min at 4°C. Finally the pellet was either gently

resuspended in 1.5 mL regeneration broth containing 0.5 M, 1 M or 1.2 M sucrose or in 200 µL ice cold 1× STC and then adding 1.3 mL regeneration broth containing 0.5 M, 1 M or 1.2 M sucrose.

Protoplasts in regeneration broth were incubated for 4, 16, or 24 h at 25°C with shaking at 120 rpm. A volume of 50 µL from the 1.5 mL regeneration broth containing 2×10^6 protoplasts were then plated on ½ PDA (1.2% agar) medium. Petri dishes were incubated at 25°C for 2 weeks. The number of regenerated protoplasts was counted. The average percentage of the regeneration frequency was determined in the presence and absence of PEG in three separate experiments.

3.2.7.2. Protoplast sensitivity to hygromycin B

The concentration of antibiotic required to inhibit the growth of non-transformed protoplasts was determined on PDA (1.2% agar) or on regeneration media supplemented with varying concentrations of hygromycin B (1-15 µg/mL).

3.2.7.3. PEG-mediated transformation of fungal protoplasts with pGpdGFP

Protoplasts were transformed using the modified procedure of Malardier *et al.* (1989). Aliquots of protoplasts in 150 µL STC were mixed with 10 µg of pDNA (pGpdGFP) (in ice cold 2× STC) and the total volume adjusted to 200 µL with ice cold 1× STC. Negative controls lacked DNA. Treatments and negative controls were incubated for 5 min on ice, mixed with 100 µL of PEG solution and incubated on ice for 20 min. The same PEG solution (1 mL) was added and gently mixed so as not to lyse the protoplasts and incubated at room temperature for 15 min. Protoplasts were then pelleted at 20000 × *g* for 2 min at 4°C and washed with 1 mL ice cold 1× STC, collected by centrifugation at 20000 × *g*, 2 min at 4°C. Finally the pellet was either gently 1) resuspended in 100 µL ice cold 1× STC, or 2) resuspended in 1.5 mL regeneration broth containing 0.5 M sucrose or 3) resuspended

in 200 µL ice cold 1× STC and then adding 1.3 mL regeneration broth containing 0.5 M sucrose.

3.2.7.4. Protoplast regeneration after transformation

Regeneration solid medium

Protoplasts and negative controls in 100 µL ice cold 1× STC from treatment 1 were plated directly on selective regeneration solid media (6 µg/mL hygromycin B). Petri dishes were incubated at 25°C for 2 weeks. Regenerated protoplasts on selective media treated with DNA will be called putative transformants in this thesis. A total of 120 putative transformants that grew on selective regeneration solid medium after 2 weeks incubation, were randomly selected and transferred to selective and non-selective ½ PDA (1.2% agar) lacking osmotic stabilizers and further incubated for 2 weeks. Stable transformants that maintained their resistance to 6 µg/mL of hygromycin B were further subcultured onto 25, 50 and 100 µg/mL hygromycin B and incubated at 25°C for 1 week. Putative transformants that grew on non-selective ½ PDA (1.2% agar) were transferred to ½ PDA (1.2% agar) containing 6 µg/mL of hygromycin B and incubated for 2 weeks. Stable transformants that maintained their resistance to 6 µg/mL of hygromycin B were further subcultured onto 25, 50 and 100 µg/mL hygromycin B and incubated at 25°C for 1 week.

Putative transformants growing on selective regeneration solid media were also examined using an agar overlay (cooled 10 mL of ½ PDA with 1.2% agar lacking hygromycin) by pouring the agar overlay onto colonies on selective regeneration media two weeks after transformation.

Regeneration broth

Protoplasts and negative controls in 1.5 mL regeneration broth in treatment 2 and 3 were incubated for 4 h at 25°C with shaking at 120 rpm to enable protoplast regeneration and

then plated on ½ PDA (1.2% agar) supplemented with hygromycin B (6, 25, 50 and 100 µg/mL). Petri dishes were incubated at 25°C for 2 weeks. The number of putative transformants that grew on selective PDA plates was counted and the regeneration frequency was obtained from three independent experiments.

White small putative transformants that grew on selective PDA medium were randomly selected and transferred to selective PDA medium (½ PDA containing 1.2% agar and supplemented with 25, 50 or 100 µg/mL hygromycin B) and non-selective ½ PDA (1.2% agar) medium. Colonies that grew on non-selective ½ PDA (1.2% agar) medium were transferred to 6 µg/mL hygromycin B.

Dark brown large putative transformants that grew on selective PDA medium were all selected and transferred to selective PDA medium (½ PDA containing 1.2% agar and supplemented with 25, 50 or 100 µg/mL hygromycin B).

3.2.8. Mitotic stability of hygromycin resistant phenotypes

Putative transformants generated by PEG-mediated transformation were streaked on ½ PDA (2.2% agar) plates supplemented with 25 µg/mL hygromycin B to obtain single separated colonies. A single separate colony from each putative transformant was subcultured every three days on ½ PDA (1.2% agar) plates lacking hygromycin and then replicated on ½ PDA (1.2% agar) plates containing 100 µg/mL hygromycin B. The latter was used to determine in which transfer the putative transformant lost its hygromycin B resistance. Putative transformants were classified mitotically stable if they remained resistant to hygromycin B after seven subcultures on medium without hygromycin B.

3.2.9. Confirmation of transformation by Southern hybridisation

3.2.9.1. Extraction of fungal genomic DNA

For DNA isolation, spore suspensions were prepared by scraping mycelia from a 4 day to 2 weeks ½ PDA (2.2% agar) plate into 5 mL sterile distilled water and vortexed for 1 min. A volume of 500-1000 µL of the clear spore suspension from the bottom of the bottle (as mycelia and chlamydospores tend to float) was dispensed and grown in a 10 mL starter culture (PDB) for 48 h, 120 rpm, at 25°C in the presence or absence of 25 µg/mL hygromycin. Two mL of the starter culture were inoculated into 200 mL PDB and grown in the presence or absence of 25 µg/mL hygromycin for five days at 25°C with shaking at 120 rpm. Mycelia were harvested using sterile Miracloth (Calbiochem), washed with cold sterile milliQ water and blotted dry thoroughly with unbleached paper towel (Kimberly-Clark, Australia) The dried mycelia were wrapped in aluminium foil, dropped in liquid nitrogen for 5 min, lyophilized (freeze dried) for 16-20 h and ground to a fine powder in a mortar and pestle. The mycelial powder was then aliquotted at 100 mg in 2.0 mL microtubes, and genomic DNA was extracted using the method of Andrianopoulos and Hynes (1988) with a modified extraction buffer (Table 3.1). DNA pellets were resuspended in 400 µL of 1× TE buffer and allowed to dissolve overnight at 4°C and kept at -20°C.

3.2.9.2. Southern hybridisation

Restriction digests

Mycelia for DNA isolation were selected from a range of hygromycin resistant *T. basicola* putative transformants, using the wild-type as a control. Approximately 1 µg of each DNA sample was digested with each of the restriction enzyme *Nhe*I or *Xba*I (Genesearch New England Biolabs) under conditions specified by the manufacturer. *Nhe*I does not cut within pGpdGFP, whereas *Xba*I has a unique site in the plasmid. The digests were allowed to incubate and after 18 h the reaction was stopped with 2 µL gel loading dye (Table 3.1).

The digests were run on 0.8% agarose gel at 38 volts for 16 h along side Lambda *Hin*d III KB standards. The migration of each Lambda *Hin*d III band away from the well was measured in cm using a ruler.

Transfer of DNA from agarose gel to membrane

The DNA was then transferred to positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by Southern blot transfer (Sambrook and Russell, 2001). Hybridisation and detection using DIG-labelled probes were performed following the manufacturer's instructions (Roche Diagnostics).

Preparation of DNA probe for use in Southern hybridisation

Southern blot membranes were probed with 1 µg of non-radioactive DIG-labelled DNA. The plasmid pGpdGFP used as a probe was prepared by digestion with the restriction enzyme *Not*I (Genesearch New England Biolabs), which has a unique site in the plasmid. Digestions were performed under the reaction conditions recommended by the manufacturer and followed by DNA purification using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

Non-radioactive DIG-labelling of DNA

The pGpdGFP plasmid (1 µg), linearised by the restriction enzyme *Not*I, was then denatured by boiling for 10 min followed by rapid cooling on ice. Using the denatured DNA as a template, random hexanucleotides were used to prime the synthesis of the probe, following the manufacturer's instructions (DIG-labelling kit, Roche Diagnostics). During the synthesis reaction, DIG, conjugated to dUTP, is incorporated into the newly synthesized strand.

3.2.9.3. Agarose gel electrophoresis

Agarose gel electrophoresis was used for fractionation of digested DNA, quantification of DNA concentration in a solution, and/or analysis of DNA fragments (size, restriction mapping). DNA samples (with added gel-loading dye) were electrophoresed on a 0.8% agarose gel (agarose in 1× TEAC buffer) (Table 3.1). Electrophoresis was performed at either 30 or 80 volts, depending on the application. For quantification and band size determination, Lambda-*Hind* III standards (Promega) were run on the same gel. Gels were stained in 2 µg/mL ethidium bromide in 1× TEAC buffer (Table 3.1) solution for visualization under ultra-violet (UV) light. Images were captured with the use of the software Grab-It (version 2.5; Synoptics Ltd., UK).

3.3. Results

3.3.1. Preparation of fungal protoplasts

3.3.1.1.Germination of endoconidia

Microscopic examination of endoconidia harvested following incubation for different periods of time (0 to 24 h) revealed that the initiation of the germination of the *T. basicola* endoconidia was observed after incubating endoconidia in PDB for 2 h at 25°C (Figure 3.2).

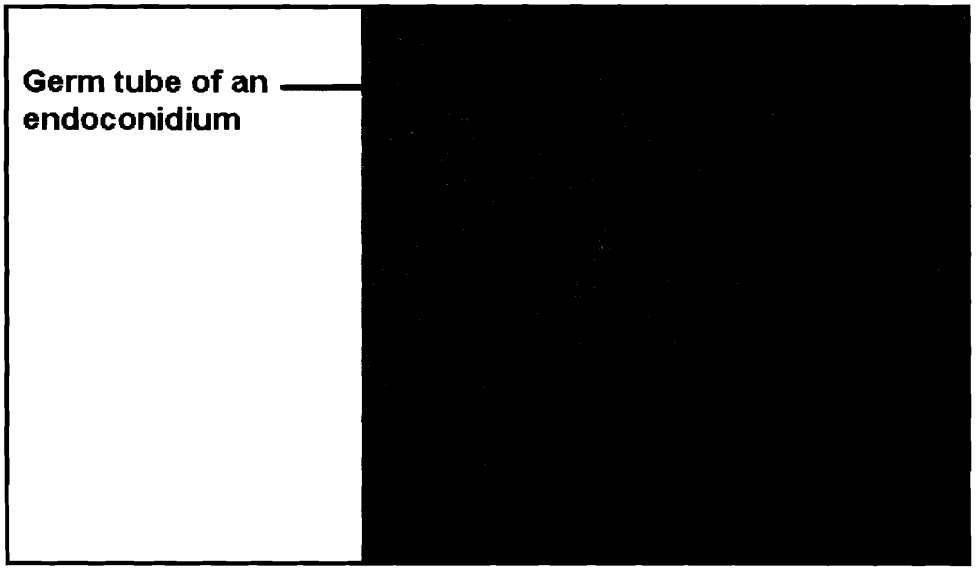

Figure 3.2. Germination of endoconidia of *Thielaviopsis basicola*

The appearance of *T. basicola* endoconidia at 2 h incubation in PDB at 25°C. Germination was initiated within 2 h and germ tubes were produced from endoconidia.

3.3.1.2. Effect of lysing enzyme on protoplast production

Initial attempts to optimize the concentration of Glucanex required for releasing protoplasts from germinating endoconidia of *T. basicola* revealed that out of four different Glucanex concentrations (5, 10, 25 and 50 mg Glucanex/mL of osmotic medium) 5 mg/mL was the most optimal. A concentration of 5 mg/mL of Glucanex in osmotic medium released protoplasts, yielding approximately 1×10^7 protoplasts/mL from 0.3-0.5 g wet

weight of germinating endoconidia (Figure 3.3). Higher concentrations of Glucanex had negative effects destroying large numbers of cells and protoplasts.

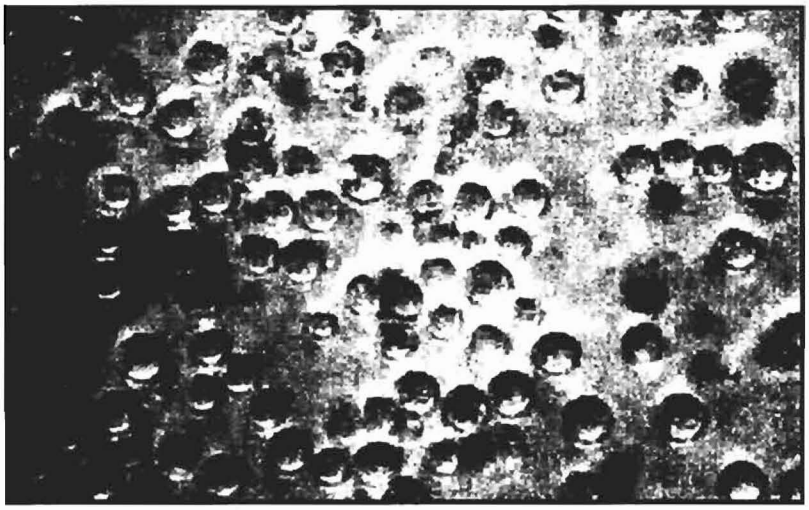

Figure 3.3. *Thielaviopsis basicola* protoplasts

Appearance of *T. basicola* protoplasts following 2 h incubation in 5 mg/mL of Glucanex in osmotic media.

3.3.1.3. Optimizing protoplast regeneration media and determining true protoplasts

To determine the optimal conditions for regenerating protoplasts on regeneration solid media and to determine true protoplasts, the regeneration of protoplasts diluted in 1:1000 with ice cold 1× STC and plated on non-selective regeneration solid media (1 M or 1.2 M sucrose) was successful. Tiny white colonies appeared 3 days after incubation (Figure 3.4 A) on both concentrations of osmotic stabilizers. The colonies showed reduction in mycelial growth and colony diameter reaching approximately 0.5 mm. They continued to grow on regeneration media and established mycelia after 7-10 days incubation. The colonies were then transferred to ½ PDA (1.2% agar) plates lacking osmotic stabilizers. After 3 days incubation, they germinated readily and rapidly, hyphal structure was healthy and displayed a normal phenotype. The regeneration of protoplasts was similar both in the presence of 1 M sucrose or 1.2 M sucrose, therefore, it was decided to continue the regeneration of protoplasts using the osmotic stabilizer 1 M sucrose.

Protoplasts that were diluted in 1: 1000 with sterile distilled water showed no growth on regeneration medium (Figure 3.4 B). Therefore, protoplasts diluted in 1000 µL ice cold 1× STC that formed colonies were true protoplasts.

Figure 3.4. Colonies regenerated from *Thielaviopsis basicola* protoplasts plated on regeneration medium

(A) Protoplasts diluted in 1: 1000 ice cold 1× STC grew on non-selective regeneration media 3 days after incubation. (B) Protoplasts diluted in 1:1000 distilled water did not grow on non-selective regeneration media 3 days after incubation.

3.3.2. Optimising protoplast regeneration broth and determining the effect of PEG on protoplast regeneration

Out of three different concentrations of sucrose (0.5, 1 or 1.2 M) contained in regeneration broth incubated at 25°C for three different time periods (4, 16 and 24 h), the regeneration of 2×10^6 protoplasts incubated for 4 h in regeneration broth containing 0.5 M sucrose proved to be successful. Colonies appeared on ½ PDA (1.2% agar) medium after 3 days incubation and displayed a normal phenotype similar to the wild-type. No growth was observed on PDA media containing protoplasts which were incubated in regeneration broth supplemented with 1 or 1.2 .M sucrose incubated for 4, 16 and 24 h at 25°C. There was also no growth on ½ PDA (1.2% agar) medium from protoplasts incubated for 16 or 24 h in regeneration broth containing 0.5 M sucrose.

In order to determine the effect of PEG on the regeneration of protoplasts that were not treated with 200 µL ice cold 1× STC, the percentage of regeneration frequency of protoplasts in the presence and absence of PEG was determined from an average of three experiments. The average percentage of regenerating protoplasts that grew on PDA medium from protoplasts treated with PEG was 1.6%. When PEG was not included in the treatment, the percentage of regenerating protoplasts increased to 4.8% (Figure 3.5).

In order to further increase the percentage of regenerating protoplasts, the toxic effect of PEG was further diluted by resuspending protoplasts in 200 µL ice cold 1× STC before adding 1.3 mL regeneration broth. On PDA medium, the effect of 200 µL ice cold 1× STC in the presence of PEG, increased the percentage of the regeneration frequency by five fold (from 1.6% to 8.2%). In the absence of PEG, the regeneration frequency of protoplasts increased by approximately 3 fold (from 4.8% to 13.8%) (Figure 3.5).

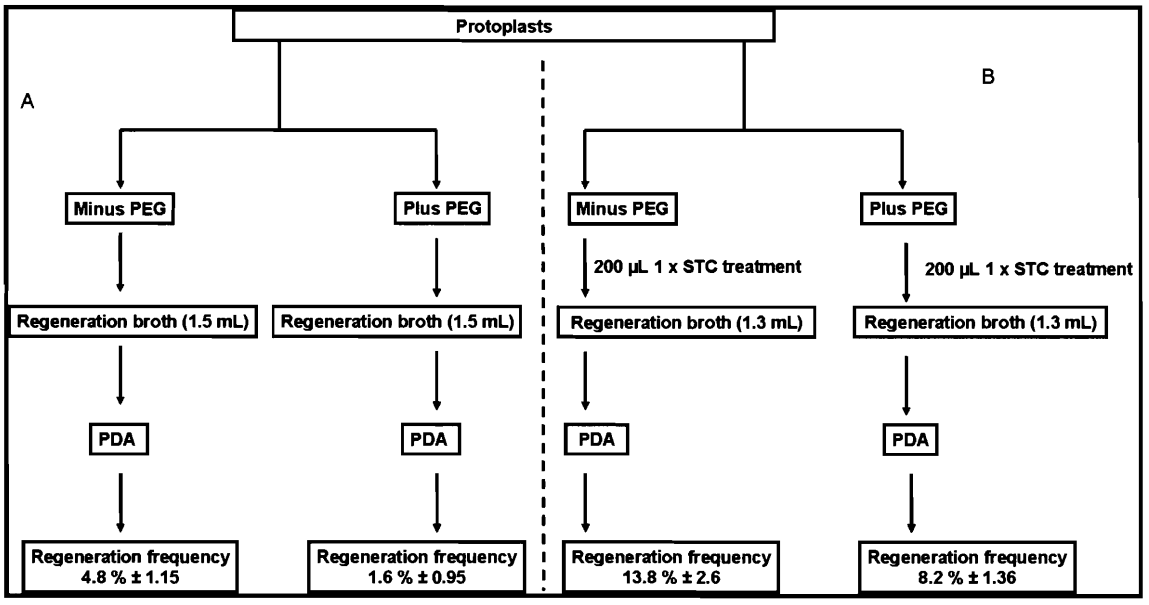

Figure 3.5. Flow chart showing regeneration frequency of *Thielaviospsis basicola* protoplasts in the presence and absence of PEG.

(A) 2 × 10^6 protoplasts in 1× STC were subjected to 1× STC (minus PEG) or PEG treatment (plus PEG) and pelleted. The pellet was then resuspended directly in 1.5 mL regeneration broth (0.5 M sucrose) and incubated for 4 h. After 3 days incubation, regeneration of colony forming units on PDA (1.2% agar) in minus PEG treatment was 4.8% and plus PEG treatment was 1.6%.
(B) In another treatment the protoplasts were subjected to the same procedure except that the pellet was first resuspended in 200 µL 1× STC to further dilute the toxic effect of PEG and then 1.3 mL regeneration broth (0.5 M sucrose) was added and incubated for 4 h. After 3 days incubation, regeneration of colony forming units on PDA (1.2% agar) in minus PEG treatment was 13.8% and plus PEG treatment was 8.2%.
(±) represents standard deviation.

3.3.3. Sensitivity to hygromycin B

To select for transformants, a suitable concentration of hygromycin B is required, upon which untransformed protoplasts do not grow. *T. basicola* protoplasts were therefore screened for their sensitivity towards hygromycin B at varying concentrations. Hygromycin B has been shown to inhibit protein synthesis by causing mistranslation (Gonzalez, 1978) and by interfering with protein translocation (Singh, 1979). In preliminary tests, wild-type protoplasts showed a high level of sensitivity to hygromycin B, requiring only 6 µg/mL (in PDA or in regeneration media containing 1 M or 1.2 M of sucrose) for inhibiting growth. The transformation experiments were carried out using 6, 25, 50 or 100 µg/mL of hygromycin B.

3.3.4. Regeneration of DNA-treated protoplasts

3.3.4.1. Regeneration solid media

Plasmid pGpdGFP was used to develop a PEG-based transformation procedure for *T. basicola*. Protoplasts treated with plasmid DNA were initially plated directly onto selective regeneration solid media (Figure 3.6). At a concentration of 6 µg/mL of hygromycin B, putative transformants appeared after 10-14 days compared to colonies of untreated protoplasts that grew within 3 days on non-selective regeneration solid media (section 3.2.6.3). The putative transformants showed reduction in mycelial growth on selective regeneration solid medium compared to colonies of untreated protoplasts. Two weeks after incubation, the putative transformants ceased to grow. No growth was observed on selective regeneration medium containing higher hygromycin B concentrations (25, 50 and 100 µg/mL). Controls not treated with DNA showed no growth on selective regeneration medium (Figure 3.6). A total of 120 putative transformants were randomly selected from selective solid regeneration medium and transferred to selective ½ PDA (1.2% agar) medium containing 6 µg/mL of hygromycin B and ½ PDA (1.2%) medium after 2 weeks incubation. On selective ½ PDA (1.2% agar) medium, only 4 out of 120 putative transformants (3.5%) grew after 5-7 days incubation (Figure 3.7 A). The other 116 were thought to be abortive transformants.

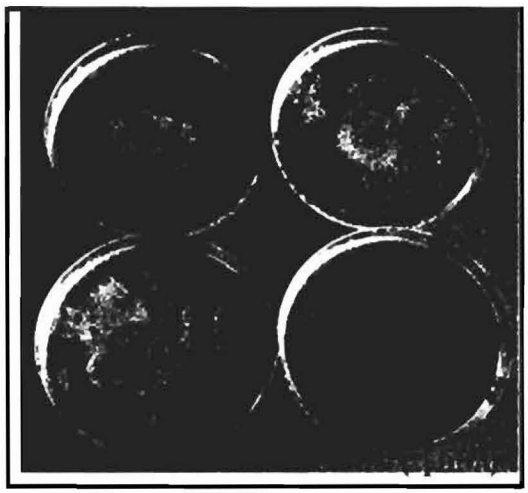

Figure 3.6. DNA-treated protoplasts plated on regeneration solid media

Plates show *Thielaviopsis basicola* putative transformants growing for 2 weeks on regeneration solid media (1 M sucrose) containing 6 µg/mL of hygromycin B. The colonies showed reduction in mycelial growth with colony diameter reaching approximately 0.5 mm. Controls not treated with DNA showed no growth on selective regeneration solid media (1 M sucrose) containing 6 µg/mL of hygromycin B.

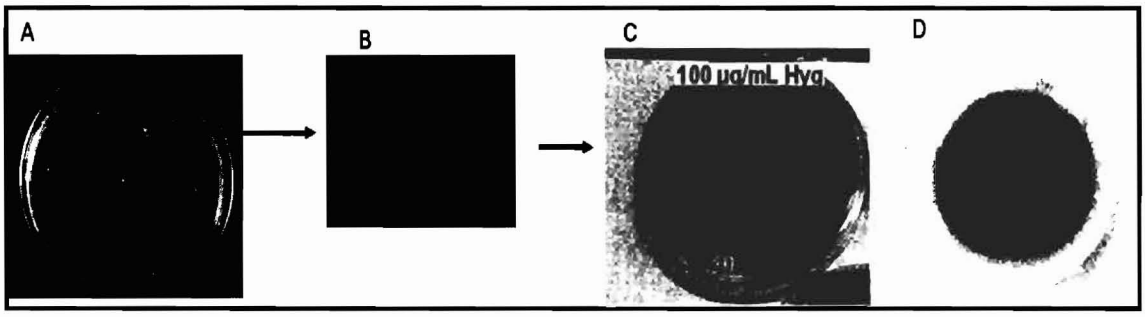

Figure 3.7. Stable and Abortive putative transformants from DNA-treated protoplasts on regeneration solid media.

(A) Putative transformants growing on PDA media containing 6 µg/mL hygromycin B 5 days after being transferred from selective regeneration media. The plate shows four large putative transformants. The small colonies ceased to grow representing abortive transformants. (B) Arrow shows mycelia and several chlamydospores when one of the large colony was observed under the stereomicroscope. During this stage, the colony and wild-type *Thielaviopsis basicola* were transferred to fresh selective PDA plates containing 100. µg/mL hygromycin B (C); Wild-type mycelia showed no growth whereas large colonies showed mycelial growth similar to wild-type *T. basicola* (D).

The growing colonies of the stable transformants observed under the stereomicroscope showed mycelia and several chlamydospores (Figure 3.7 B). The four stable putative transformants, when further transferred to fresh selective PDA media containing 25, 50 and

100 µg/mL hygromycin B, grew displaying a normal phenotype similar to the wild-type (Figure 3.7 C&D). Thus, approximately, 96.5% (116/120) of the putative transformants in the three experiments were unable to grow after subculturing on selective PDA media containing 6 µg/mL of hygromycin B after 2 weeks incubation, representing abortive transformants (Figure 3.8). On non-selective PDA media, all the 120 putative transformants grew displaying a normal phenotype similar to the wild-type. Further replication onto selective PDA media generated the same four hygromycin B resistant stable transformants.

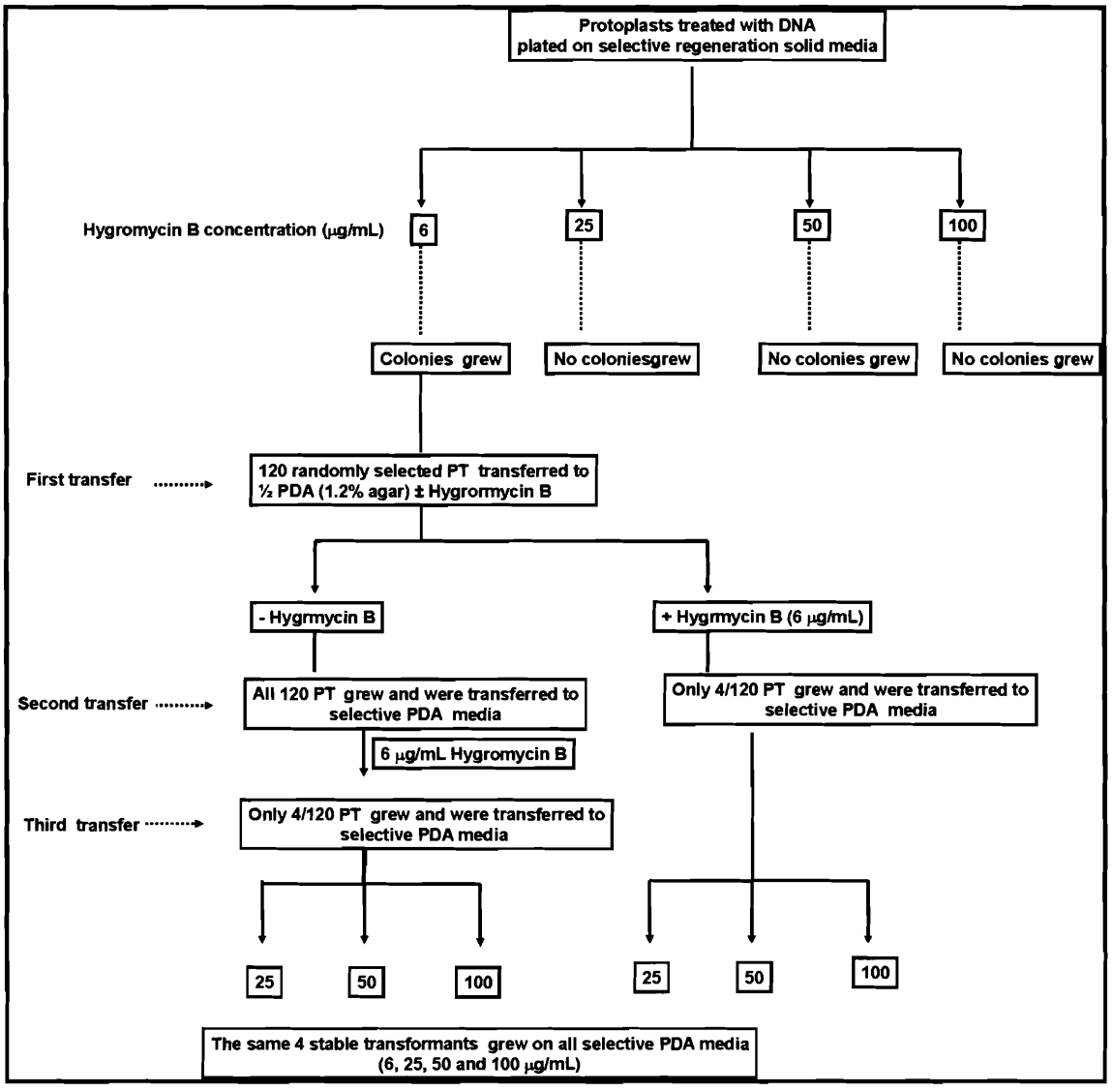

Figure 3.8. Flow chart showing *Thielaviopsis basicola* protoplasts treated with DNA on selective regeneration media

DNA treated protoplasts in the presence of PEG resuspended in 100 µL 1X STC were plated on solid selective regeneration solid media. Growth occurred on PDA media containing 6 µg/mL of hygromycin B. No growth occurred on PDA media containing 25, 50 and 100 µg/mL of hygromycin B. Random selection of 120 PT (putative transformants) onto selective and non-selective PDA media yielded approximately 96.5% (116/120) abortive transformants and 3.5% (4/120) stable transformants that were resistant up to 100 µg/mL of hygromycin B

The average transformation frequency from the three independent experiments was 0.13% per μg of DNA (Table 3.2)

Table 3.2. Transformation frequency of protoplasts regenerated on regeneration solid media

Experiment	Amount of pDNA used	Number of putative transformants	Transformation frequency/μg DNA
1	10 μg	1	0.1
2	10 μg	1	0.1
3	10 μg	2	0.2
			Average 0.13

Number of *Thielaviopsis basicola* stable transformants regenerated on selective solid media in three independent experiments. The transformation frequency was calculated as the number of transformants per microgram of plasmid DNA.

3.3.4.2. Regeneration broth

Studies suggest that transformation frequencies may be significantly increased by allowing protoplasts to recover following transformation initially in osmotically stabilized liquid media (regeneration broth) rather than plated directly on solid media (regeneration media), thereby allowing expression of the hygromycin resistant phenotype (Inglis *et al.*, 1999). DNA-treated protoplasts incubated in 1.5 mL regeneration broth for 4 h grew on selective PDA (Figure 3.9).

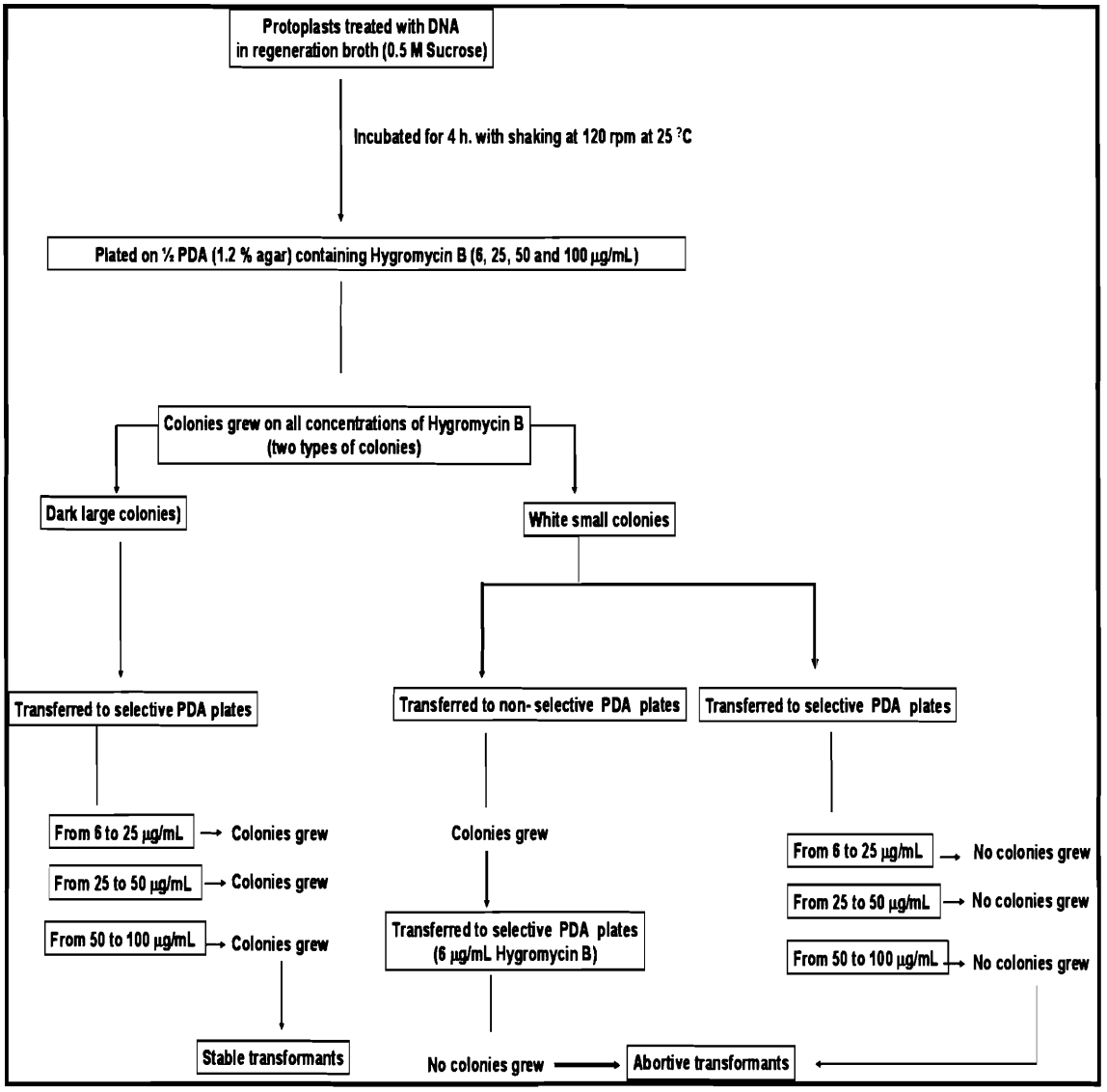

Figure 3.9. Flowchart of protoplasts treated with DNA resuspended in regeneration broth.

DNA treated protoplasts of *Thielaviopsis basicola* in the presence of PEG resuspended directly in regeneration broth (treatment 1) or in 200 µL 1× STC (treatment 2) before resuspending in regeneration broth yielded approximately >99% abortive transformants and <1% stable transformants that were resistant up to 100 µg/mL of hygromycin B

In order to determine the regeneration frequency, putative transformants that grew on selective PDA media were counted and their percentage from the initial number of protoplasts (2×10^6) used was calculated. It was found that the percentage of total putative transformants was 0.09% at 6, 0.06% at 25, 0.04% at 50 and 0.03% at 100 µg/mL hygromycin B (Table 3.3). The percentage of putative transformants decreased with an increase in the hygromycin B concentration.

Table 3.3. The effect of regeneration broth on the average number of *Thielaviopsis basicola* stable transformants.

Hygromycin conc. (µg/mL)	Number of putative transformants out of 2×10^6 initial protoplasts	% of putative transformants out of 2×10^6 initial protoplasts	% of Abortive transformants	*Average Number of stable transformants
6	1882 ± 441	0.09 ± 0.02	99.6 ± 0.4	0.7 ± 0.6
25	1227 ± 315	0.06 ± 0.01	99.3 ± 0.8	1.0 ±1.0
50	840 ± 211	0.04 ± 0.01	99.2 ± 0.8	1.0 ± 1.0
100	637 ± 195	0.03 ± 0.01	99.0 ± 1.0	1.0 ± 1.0

Number of *T. basicola* surviving protoplasts from 2×10^6 initial protoplasts treated with 10 µg DNA placed in 1.5 mL regeneration broth (0.5 M sucrose PDB, 4 h incubation, and then plated onto ½ PDA (1.2% agar) with hygromycin in three independent experiments. (±) represents standard deviation of the mean.
* numbers are per 2×10^6 initial protoplasts treated with 10 µg of DNA.

Approximately 99% of the putative transformants ceased to grow any further on selective PDA media 14 days after transformation, representing abortive transformants (Table 3.3). They appeared as white colonies reaching a size of approximately 5 mm in diameter. Approximately less than 1% of the putative transformants displayed larger healthy mycelial colonies similar in appearance to the wild-type grown without hygromycin B and were termed stable transformants (Figure 3.10). The stable transformants that were readily visible on 6, 25, 50 and 100 µg/mL hygromycin B, increased in diameter every 24 h and had to be replated to prevent overlapping of mycelia. Stable transformants that grew at lower concentrations of hygromycin B were transferred to higher concentrations and showed growth (Figure 3.9). The higher number of putative transformants did not have a major effect in increasing the production of stable transformants. For example, the regeneration percentage rate of putative transformants at 0.09% produced an average number of 0.66 stable transformants, but at a lower survival percentage rate of 0.04%, an average number of 1 stable transformant was produced.

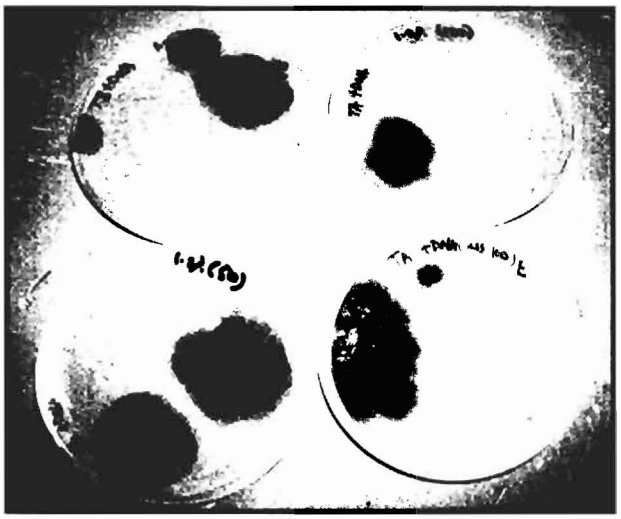

Figure 3.10. *Thielaviopsis basicola* transformants in regeneration broth plated on PDA media (1.2% agar)

Putative transformants plated on selective PDA media containing various concentrations of hygromycin B (6, 25, 50 and 100 µg/mL) after incubation for 4 h in 200 µL 1X STC and regeneration broth containing 0.5 M sucrose. Two types of colonies can be seen; large brown colonies termed stable transformants and small white colonies termed abortive transformants.

There was also no major effect of the hygromycin concentration on the average number of stable transformants produced. For example, the average number of stable transformants produced at 6 µg/mL hygromycin B was 0.66 whereas at 25, 50 or 100 µg/mL hygromycin B, the average number produced was 1 stable transformant. The transformation frequency of stable transformants obtained was 0.27 per µg of DNA (Table 3.4).

Table 3.4. Transformation frequency of *Thielaviopsis basicola* protoplasts regenerated directly in regeneration broth

Experiment	Amount of pDNA used	Number of stable transformants	Transformation frequency/µg DNA
1	10 µg	4	0.40
2	10 µg	2	0.20
3	10 µg	2	0.20
			Average 0.27

Number of *T. basicola* stable transformants regenerated in regeneration broth (0.5M sucrose PDB, 4 h incubation) before being plated on selective solid media in three independent experiments. The transformation frequency was calculated as the number of transformants per microgram of plasmid DNA.

To further investigate whether the abortive transformants could be enhanced in their growth and resistance to hygromycin B, a total of 75 colonies were randomly transferred

onto non-selective PDA media and selective PDA media. Healthy looking colonies similar to the wild-type grew on non-selective media but no growth was observed on selective PDA media. Those that grew on non-selective PDA media were further subcultured onto selective PDA media but no growth was observed (Figure 3.9 & Figure 3.11). Controls not treated with DNA showed no growth on selective PDA media.

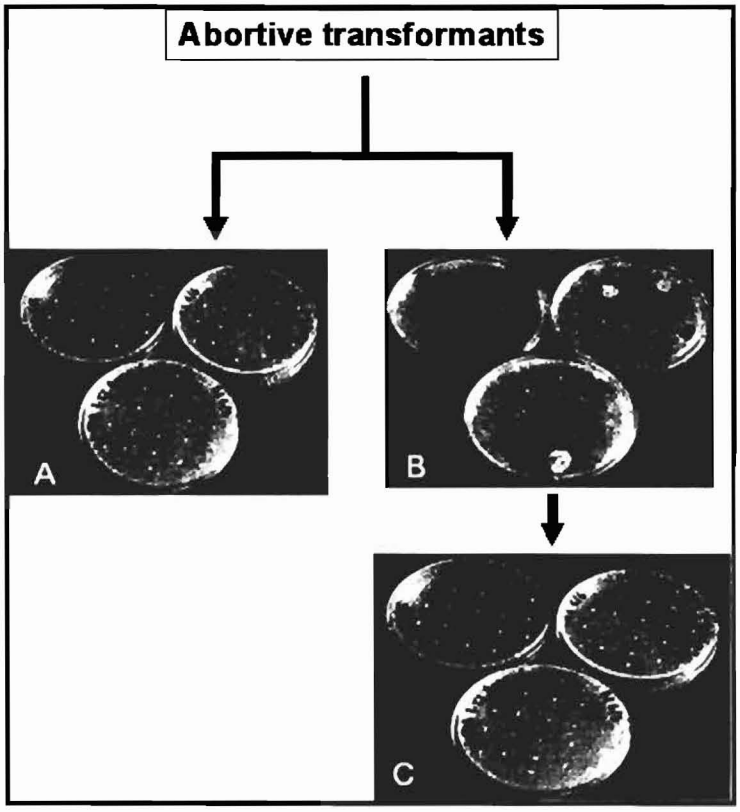

Figure 3.11. *Thielaviopsis basicola* abortive transformants

Abortive transformants showed no growth when subcultured directly onto selective PDA media containing 6 µg/mL hygromycin B (A), but showed growth when subcultured directly onto non-selective PDA media (B). When subcultured from non-selective PDA media to selective PDA media containing 6 µg/mL hygromycin B, they did not grow (C).

3.3.3.3. Effect of resuspending the DNA-treated protoplast pellet in 200 µL 1× STC and regeneration broth

In order to increase the number of stable transformants, the toxic effect of PEG was further diluted by resuspending the DNA treated protoplast pellet in 200 µL 1× STC and in 1.3 mL regeneration broth. On selective PDA media, the percentage of putative transformants

increased to almost 10 fold higher compared to protoplasts that were not treated in 200 µL 1× STC. The influence of diluting the toxic effect still generated more than 99% abortive transformants and less than 1% stable transformants (Table 3.5). However, the transformation frequency of stable transformants increased also by approximately 10 fold (from 0.27 to 2.5 per µg of DNA) (Table 3.6).

There was a major effect of the concentration of the hygromycin B at 25 µg/mL on the average number of stable transformants obtained compared to 6, 50 and 100 µg/mL. At the lowest concentration of hygromycin B (6 µg/mL) only an average of 1 stable transformant was obtained. The highest average number of transformants was obtained at 25 µg/mL hygromycin B. There was no major difference at the average number of transformants obtained at 50 and 100 µg/mL of hygromycin B) (Table 3.5). Therefore, the transformation experiments were carried out using the resuspension of DNA-treated protoplast pellet in 200 µL 1× STC and 1.3 mL regeneration broth and 25 µg/mL of hygromycin B in the final protocol.

Table 3.5. The effect of 200 µL 1× STC treatment on the average number of *Thielaviopsis basicola* stable transformants

Hygromycin conc. (µg/mL)	Number of transformants out of 2×10^6 initial protoplasts	% of transformants out of 2×10^6 initial protoplasts	% of Abortive transformants	*Average Number of Stable transformants
6	17925 ± 4865	0.9 ± 0.2	99.9 ± 0	3.0 ± 0
25	14795 ± 4445	0.7± 0.2	99.3 ± 0.3	14.7 ± 8.6
50	12660 ± 3842	0.6 ± 0.2	99.7 ± 0.1	5.0 ± 1.7
100	10738 ± 2860	0.5 ± 0.1	99.7 ± 0.1	4.0 ± 0

Number of *T. basicola* surviving protoplasts from $2X10^6$ initial protoplasts treated with 10 µg DNA resuspended in 200 µL 1× STC before being placed in 1.3 mL regeneration broth (0.5 M sucrose PDB, 4 h incubation, and then plated onto ½ PDA (1.2% agar)with hygromycin). in three independent experiments. (±) represents standard deviation of the mean.
* numbers are per 2×10^6 initial protoplasts treated with 10 µg of DNA.

Table 3.6. Transformation frequency of *Thielaviopsis basicola* protoplasts resuspended in 200 µL 1× STC and regenerated in regeneration broth

Experiment	Amount of pDNA used	Number of stable transformants	Transformation frequency/µg DNA
1	10 µg	15	1.5
2	10 µg	22	2.2
3	10 µg	37	3.7
			Average 2.5

Number of *T. basicola* stable transformants regenerated in regeneration broth (0.5M sucrose PDB, 4 h incubation) and 200 µL 1× STC before being plated on selective solid media in three independent experiments. The transformation frequency was calculated as the number of stable transformants per microgram of plasmid DNA.

3.3.5. Mitotic stability of putative transformants

Following 7 subcultures on PDA medium, 90.5% transformants retained their ability to grow on selective ½ PDA (1.2% agar) medium supplemented with 100 µg/mL of hygromycin B and were classified as mitotically stable transformants. 9.5% demonstrated sensitivity to hygromycin B during different transfer stages and were classified as mitotically unstable transformants. The unstable transformants maintained their hygromycin B resistance under selective pressure. All transformants grown on selective or non-selective media never formed sectors.

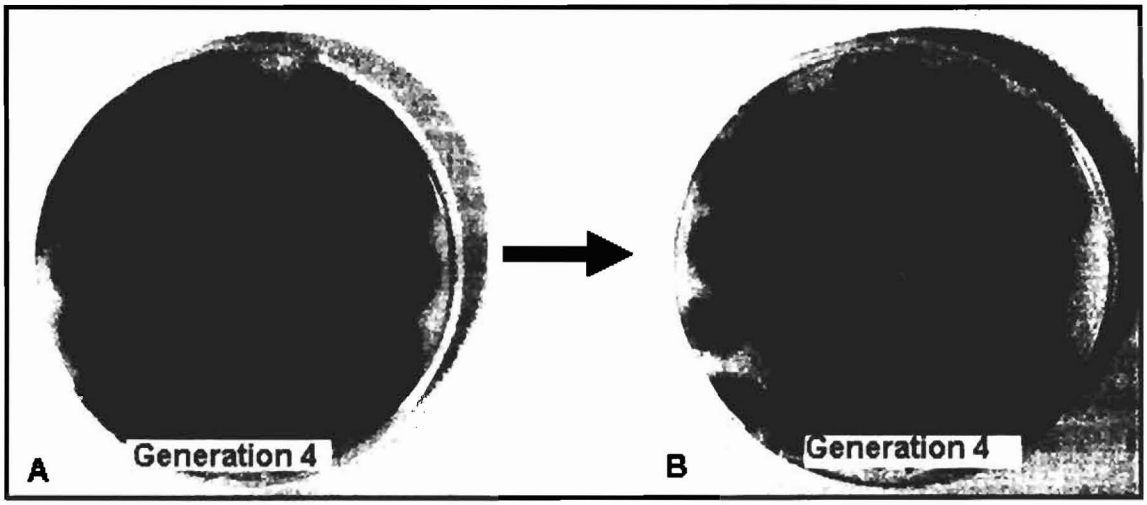

Figure 3.12. Mitotic stability of *Thielaviopsis basicola* transformants

(A): shows generation 4 of putative transformants on non-selective media. (B) shows generation 4 on selective media (100 µg/mL hygromycin). Note three putative tansformants lost their hygromycin B resistant phenotype and were termed mitotically unstable transformants. Two are indicated by red arrows.

3.3.6. Confirmation of transformation by Southern hybridisation analysis

The fate of the transforming DNA was investigated for six putative transformants; four randomly selected mitotically unstable transformants (that lost their resistance to hygromycin B during subculture), one randomly selected mitotically stable transformant (that maintained its resistance to hygromycin B during subculture) and one randomly selected abortive transformant. For the unstable transformants, the genomic DNA was extracted before and after they lost their mitotic stability. The extracted genomic DNA was digested with *Nhe*I and analysed by Southern hybridisation.

Mitotically unstable transformants

Southern hybridisation analysis showed that the probe hybridised to *Nhe*I-genomic DNA of transformants before they lost their resistance to hygromycin B indicating integration of the plasmid pGpdGFP into the fungal genomes of T1, T2, T3 and T4 (Figure 3.13, lanes 2, 4, 6 and 8). The probe, however, did not hybridise to *Nhe*I-digested genomic DNA of transformants after they lost their resistant hygromycin B phenotype (Figure 3.13, lanes 3, 5, 7 and 9).

Mitotically stable transformant

The probe also hybridised to the *Nhe*I-digested genomic DNA stable transformant T5 that was grown in the presence (Figure 3.12, lane 10) and absence (Figure 3.13, lane 11) of hygromycin B, indicating that the plasmid pGpdGFP integrated into the fungal genome at a site that was stable in the fungal chromosome.

Abortive transformant

There was no hybridisation of the probe detected in the abortive transformant (Figure 3.13, T6; lane 12) indicating that the plasmid had not integrated into the fungal genome of the abortive transformant.

Wild-type

Hybridisation of the probe to the wild-type genomic DNA (lane 1) was not evident.

Hybridisation patterns

Hybridisation patterns showed that in most transformants, plasmid pGpdGFP appeared to have integrated into the genome at a single site and also showed that the hybridizing bands are of different sizes, indicating that the plasmid pGpdGFP may or may not have integrated at different chromosomal locations. Only one transformant out of five tested (Figure 3.13, lane 4) showed possible multiple integration. T3 (Figure 3.13, lane 6) and T4 (Figure 3.13, lane 8) showed strong hybridisation signals suggesting the integration of more than one copy of the plasmid in tandem.

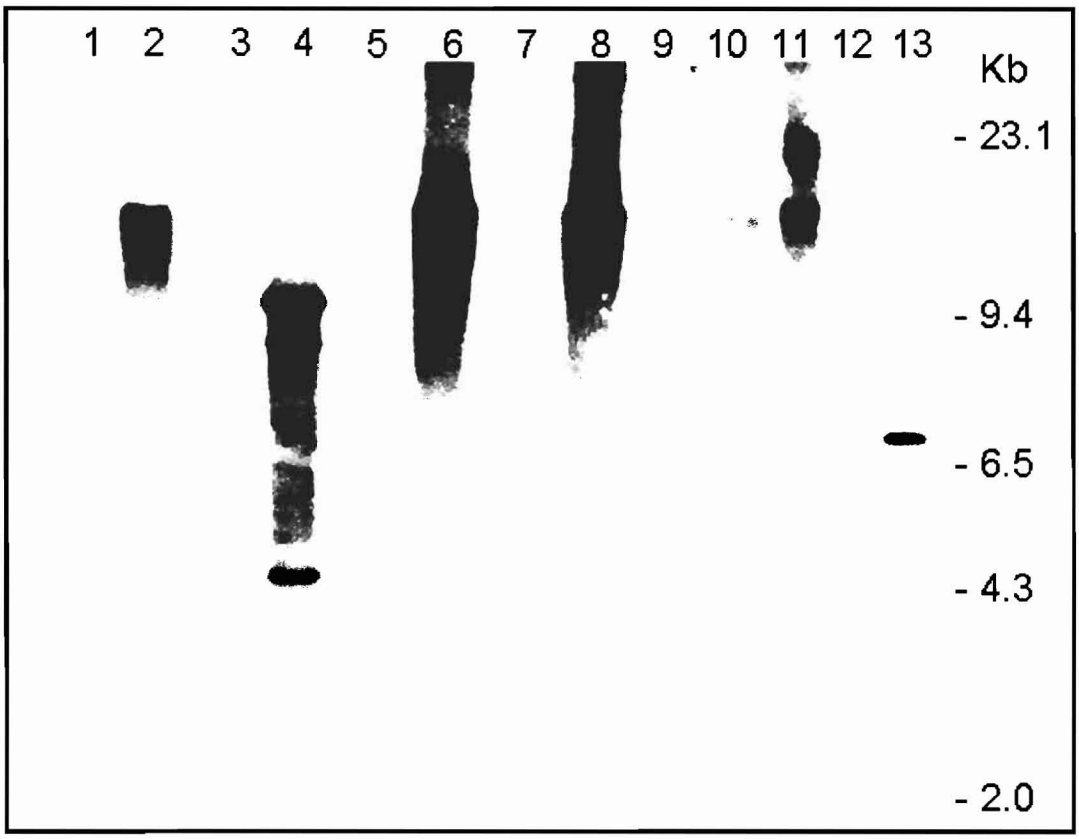

Figure 3.13. Southern hybridisation analysis of *Nhe*I-digested genomic DNA from *Thielaviopsis basicola* transformants

T. basicola was transformed with pGpdGFP. *Nhe*I does not cut pGpdGFP. Wild-type (BRIP40192 isolate) (Lane 1), four randomly chosen mitotically unstable transformants that showed resistance to hygromycin B; T1 Hyg^R (lane 2), T2 Hyg^R (lane 4), T3 Hyg^R (lane 6) and T4 Hyg^R (lane 8) but later lost their resistance during different transfers (($T1\ Hyg^s$ (lane 3), $T2\ Hyg^s$ (lane 5), $T3\ Hyg^s$ (lane 7) and $T4\ Hyg^s$ (lane 9), one randomly selected mitotically stable transformant grown in the presence of hygromycin B ; $T5\ Hyg^R$ +Hyg (lane 10) and in the absence of hygromycin B; $T5\ Hyg^R$ – Hyg (lane 11) and one randomly selected abortive transformant ; $T6\ Hyg^S$ (lane 12). Lane 13; plasmid pGpdGFP linearized with *Xba*I (positive control).

Equivalent amounts of DNA were loaded in all lanes and fractionated in 0.8% agarose gel at 38 V. Southern hybridisation analysis was carried out as described in section 3.2.9.2. Digoxigenin (DIG)-labeled plasmid pGpdGFP linearised with *Not*I was used as a probe. Some fragments of the molecular size marker Lambda (λ) *Hin*d III are indicated in kilobases on the right.

To verify the fate of the transforming DNA into the unstable transformants, the undigested DNA from each of the unstable transformant before they lost their resistance to hygromycin B (Figure 3.14; lane 2, 4, 6 and 8) was electrophoresed, transferred onto nylon membrane, and hybridised using the plasmid pGpdGFP as a probe. The pGpdGFP probe hybridised to high molecular weight (23.1 kb) undigested DNA (Figure 3.14; lane 2, 4, 6 and 8), suggesting that the plasmid integrated into the genome and did not replicate autonomously. No hybridisation to fast-migrating DNA, corresponding to free plasmid was observed. This result was consistent with the undigested stable transformants used as a positive control (Figure 3.14; lane 10).

To determine the copy number of pGpdGFP per genome, the genomic DNA was digested with *Xba*1 which cuts the inserted pGpdGFP only once. More than two hybridizing bands were observed in all transformants digested with *Xba*I except for T5 (lane 9), indicating that there were likely more than two copies of the plasmid inserted in the genomic DNA. Transformants, T3 (Figure 3.14, lane 5) and T4 (Figure 3.14, lane 7) showed strong hybridisation signals corresponding to the molecular weight of the plasmid pGpdGFP (Figure 3.14, lane 12), suggesting the integration of more than one copy of the plasmid in tandem. Hybridising fragments larger and smaller than 6.9 kb probably corresponded to DNA in the flanking regions of the integration sites.

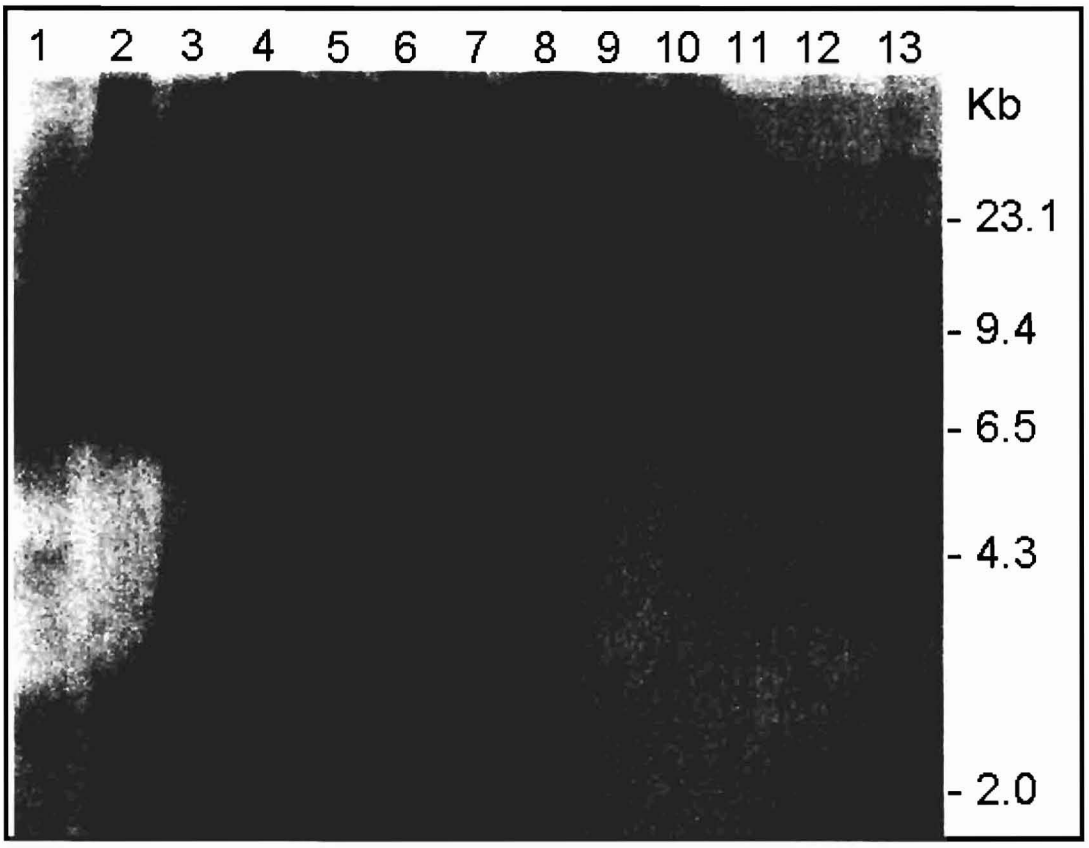

Figure 3.14. Southern hybridisation analysis of *Xba*I-digested and undigested genomic DNA from *Thielaviopsis basicola* unstable transformants.

T. basicola was transformed with pGpdGFP. *Xba*I cuts once in pGpdGFP. Four unstable transformants digested with *Xba*I; T1 (lane 1), T2 (lane 3), T3 (lane 5) and T4 (lane 7) and undigested; T1 (lane 2), T2 (lane 4), T3 (lane 6) and T4 (lane 8) and one stable transformant digested with *Xba*I; T5 (lane 9)). Controls; lane 10 (undigested; T5), lane 11 (untransformed wild-type *T. basicola*, BRIP40192 isolate), lane 12 (plasmid pGpdGFP linearized with *Xba*I) and lane 13 (uncut pGpdGFP).

Total DNA undigested and digested with *Xba*I were loaded in equivalent amounts in all lanes and fractionated in 0.8% agarose gel at 38 V. Southern hybridisation analysis was carried out as described in section 3.2.9.2. Digoxigenin (DIG)-labeled plasmid pGpdGFP linearised with *Not*I was used a probe. Some fragments of the molecular size marker Lambda (λ) *Hin*d III are indicated in kilobases on the right.

3.4. Discussion

Although transformation systems have been developed for some filamentous fungi with the aim of identifying pathogenicity genes, developing a transformation system for a new species can be technically difficult. PEG-mediated transformation has been previously reported for a wide variety of fungal species, however, successful implementation of this technology had not yet been applied in *T. basicola*. In this study a protocol for PEG-mediated transformation of *T. basicola* was developed.

PEG-mediated transformation requires the production of protoplast. Protoplasts have been obtained from all the major taxonomic groups of fungi, however, their production varies from species to species and even between isolates (Robinson and Deacon, 2001). To our knowledge, no protoplast systems have ever been developed for the genus *Thielaviopsis*. Although the literature describes several protocols for obtaining protoplasts from different fungal species, and the availability of several commercial enzymes has facilitated the release of protoplasts, each fungus or fungal group will usually require its own specific conditions (Lalithakumari, 1996). In this study, conditions for preparing and regenerating protoplasts of *T. basicola* were investigated in which most of the parameters described to be important in protoplast production were tested.

Three days old cultures were used for harvesting endoconidia, because it was reported that protoplast yield decreases with aging of the culture as the fungal cell walls tend to resist lysing (Peberdy and Ferency, 1985). Endoconidia were selected for producing protoplasts as they are more likely to yield a homogenous suspension of mononucleate cells, which are more suitable for transformation than mycelia (Peberdy and Ferency, 1985). They are also much easier to handle than chlamydospores in producing protoplasts due to the presence of melanin in chlamydospores. Melanin has been reported to inhibit the action of lytic

enzymes during protoplasting (Peberdy and Ferency, 1985). A pre-incubation step of endoconidia in PDB is necessary as this enables the endoconidia to swell or germinate and this stage has been reported to facilitate the digestion of cell walls (Peberdy and Ferency, 1985)

One of the crucial steps in PEG-mediated transformation is the production of sufficient amount of protoplasts. Hydrolytic enzymes play a key role in protoplasts production. The hydrolysis of the fungal cell wall can vary with the concentration of the lytic enzyme used. Several studies have reported that combinations of enzymes act synergistically to enhance cell wall degradation and produce protoplasts (Tilburn *et al.*, 1983; Stasz *et al.*, 1988; Solis *et al.*, 1996). In contrast, Robinson and Deacon (2001) observed that out of the 10 enzyme combinations applied to *Rhizoctonia solani*, the only combination that yielded protoplasts was Novozyme 234 and Maxazym CL (Robinson and Deacon, 2001). In this study, a concentration of 5 mg/mL of Glucanex was sufficient to produce an average of 1×10^7 protoplasts/mL from *T. basicola* endoconidia, which is 10 times lower than the enzyme concentration required for the successful production of protoplasts in *Venturia inequalis* mycelial material (Fitzgerald *et al.*, 2003).

The osmotic stabilizer is another important component to consider protoplast viability and regeneration. Since the cell wall composition varies among species, different stabilizers at different concentrations have been optimized for some species. Salts, sugars and sugar alcohols have been used as osmotic stabilizers for fungal protoplasts (Peberdy and Ferency, 1985). The present study initially assessed protoplast viability by direct regeneration in an osmotically stabilized medium. For protoplasts diluted in 1× STC, successful regeneration of protoplasts occurred on regeneration medium containing 1 M or 1.2 M sucrose. Protoplasts diluted with distilled water did not regenerate on either regeneration media, indicating that the protoplasts obtained were true and viable protoplasts. Regeneration was successful on both types of regeneration media, the colonies

were tiny, unhealthy and showed reduction in mycelial growth. Robinson and Deacon (2001) observed that protoplasts obtained from *Rhizoctonia solani* displayed sickly phenotypes when the regeneration media contained an unsuitable osmotic stabilizer (Robinson and Deacon, 2001).

Prior to transformation, a suitable selection strategy for transformants must be developed. The present study examined the susceptibility of protoplasts to hygromycin B. *T. basicola* isolate BRIP40192 was sensitive to low concentrations of hygromycin B on regeneration media (no growth at 6 µg/mL). Thus, hygromycin B was used as a selection system to develop a PEG / calcium chloride based transformation system for *T. basicola*. The hygromycin B gene has been used successfully in transformation of several filamentous fungi including *Neonectria galligena* (Tanguay *et al.*, 2003), *Crinipellis perniciosa* (Lima *et al.*, 2003) and *L. maculans* (Sexton and Howlett, 2001).

In this study, most colonies that appeared on selective regeneration medium displayed sickly phenotypes and demonstrated slow growth compared to colonies on non-selective regeneration media. After 2 weeks, colonies stopped growth compared to colonies on non-selective regeneration media and had to be subcultured. Approximately 96.5% of protoplasts incubated with pGpdGFP that grew on hygromycin B did not maintain resistance to the antibiotic upon subculture and were considered abortive transformants. Only 3.5% maintained resistance up to 100 µg/ mL of hygromycin B. The transformation frequency of 0.13 stable transformants per µg of DNA obtained in the preliminary results is not considered high. To improve the transformation frequency, successful regeneration must occur (Robinson and Deacon, 2001). It is possible that the selective regeneration media containing 1 M sucrose contributed to the appearance of the sickly phenotypes displayed by transformed protoplasts and thus to the low average transformation frequency

of 0.13 stable transformants per μg of DNA. The application of an overlay agar system to increase the number of stable transformants was not successful.

The application of osmotically stabilized liquid media (regeneration broth) over regeneration solid media has previously been suggested to dilute the toxic effect of PEG (Inglis *et al.*, 1999). In this study, regenerating the transformed protoplasts in regeneration broth containing 0.5 M sucrose as an osmotic stabilizer before plating on selective PDA media increased the transformation frequency of stable transformants from 0.13 per μg of DNA to 0.27 per μg of DNA and the addition of 200 μL 1× STC in regeneration broth further increased the transformation frequency of stable transformants to 2.5 per μg of DNA. However, this transformation frequency is still considered low. PEG-mediated transformation of other ascomycete fungi using other plasmids have generated transformation frequencies of stable transformants of 10-70 per μg of DNA and up to 2×10^4 per μg of DNA (Fincham, 1989).

Protoplasts regeneration frequencies have been observed to vary between species and strains, with the majority of filamentous fungal protoplasts demonstrating regeneration frequencies of 0.1-50% (Stasz *et al.*, 1988). Though, the effect of further diluting the toxic effect of PEG by resuspending protoplasts in 200 μL 1× STC osmotically stabilized liquid media increased the regeneration frequency of colonies by five fold, the percentage of abortive transformants was still more than 99% and stable transformants was less than 1%. Abortive transformants are characterised by a slow growth rate in selective media and cease growth when transferred to fresh selective media since the selectable marker is unstable during mitosis. The characteristics of abortive transformants obtained in this study were also consistent with other studies (Robinson and Deacon, 2001; Fitzgerald *et al.*, 2003).

Robinson and Deacon (2001) reported that the phenotype of abortive transformants was a result of non-integrative transformation. Using Southern hybridisation analysis to support their results they were able to show that hybridisation occurred towards non-integrated plasmid corresponding in molecular migration to the circular plasmid they used in their study. Non-integrative transformation is due to the DNA being able to replicate independently from the chromosomes; however they are subject to frequent losses during growth. Southern hybridisation analysis of abortive transformants in this study did not reveal any integration of the plasmid into the genome and no hybridisation was observed to fast-migrating DNA, corresponding to free plasmid pGpdGFP. Abortive transformants have been previously reported in a large number of PEG-mediated fungal transformation systems (Hamada *et al.*, 1994; Sanchez-Torres *et al.*, 1994; Schillberg *et al.*, 2000; Robinson and Deacon, 2001), indicating that the limiting step in transformation for many fungal species, is not the uptake of foreign DNA, but its integration into the chromosome. However, there is not sufficient molecular analysis data on the abortive transformants, to conclude this observation for *T. basicola.*

In this study, abortive transformants were unable to grow upon subculture on selective media and therefore could not be maintained under continuous selective pressure. In some fungal species, abortive transformants have been observed to produce sectors of vigorous growth which contain integrated plasmid DNA (Bowyer *et al.*, 1994; Steiner *et al.*, 1995; Barcellos *et al.*, 1998). Following 2 weeks incubation, sectors were never observed in *T. basicola* transformed with pGpdGFP.

The fate of transforming DNA vector in unstable transformants has been previously investigated in *Aspergillus nidulans* by Barcellos *et al.* (1998). Southern hybridisation analysis of uncut DNA of *A. nidulans* mitotically unstable transformants detected only the high molecular weight DNA of approximately 23 kb. Free plasmid was not detected. In

this study, Southern hybridisation analysis of mitotically unstable transformants resistant to hygromycin B also showed high molecular weight DNA of about 23 kb (Figure 3.14, lanes 2,4,6,8) indicating that pGpdGFP did not exist as a free plasmid capable of replicating autonomously. However, Southern analysis of mitotically unstable transformants that lost their hygromycin B resistance during different transfers did not reveal any integration of the plasmid into the genome suggesting that plasmid sequences were lost (Figure 3.13, lanes 3,5,79).

A detailed analysis (using CHEF hybridisation analysis) by Barcellos *et al.* (1998) showed that all their mitotically unstable transformants showed hybridisation signals at non-chromosomal region, indicating the presence of non-integrated forms of the plasmid DNA. They argued that if free plasmid occurs, it should be possible to transform the total DNA into *E. coli* and re-isolate the plasmid. But they were not able to produce any antibiotic resistant *E. coli* colonies. So they concluded that the high molecular weight of the hybridisation signals displayed on the undigested DNA as well as the presence of CHEF hybridisation bands at non-chromosomal regions support the notion that the plasmid molecules in the unstable transformants are concatenated, as a result no transformed *E. coli* cells were obtained. The hypotheses put forward to explain the presence of these non-integrated sequences were (i) they tandemly integrated and are continuously excised as multimeric forms and (ii) the plasmid sequences persist as large extrachromosomal molecules capable of inefficient replication (Barcellos *et al.*, 1998). Southern hybridisation analysis may have demonstrated tandem integration of pGpdGFP into the fungal genome of *T. basicola* unstable transformants, however it could not prove that the high molecular weight of the hybridisation signals displayed on the undigested DNA (Figure 3.14, lanes 2,4,6,8) represent non-integrated forms of the plasmid DNA at non-chromosomal regions. Therefore, it is possible that tandem copies of the integrated plasmid are continuously

excised as multimeric forms or they exist as unintegrated concatemers but there is yet no evidence to support this hypothesis.

Mitotically stable transformants can grow and continue to maintain their resistance without losing their mitotic stability on non-selective or selective media. Southern hybridisation analysis of *T. basicola* stable transformants revealed that the plasmid pGpdGFP may have integrated randomly into the fungal genome. However, the integration of the transforming plasmid DNA may have integrated as one or more copies in tandem arrays. This has also been reported by Bolker *et al.* (1995) where they observed that the phenomena of tandem array insertion of the transforming plasmid is typical of PEG-mediated transformation in *Ustilago maydis*.

Transformation in this study may not be constrained by DNA uptake but rather by the efficiency with which the plasmid DNA is integrated into the genome. Strategies for improving transformation efficiency of stable transformants not only depend on the nature of the protoplast and the regeneration media, but also depend on the nature of the plasmid or transforming DNA. In *Neurospora*, large circular plasmids have been reported to mediate transformation at a higher frequency than small and linear plasmids (Mishra, 1985). Low transformation frequency has been attributed to poor recognition of promoters of selection markers on plasmid DNA by some species of fungi. For example, the usage of pAN7-1 vector, in which the selectable marker *hph* is under the control of the *gpd* promoter of *A. nidulans*, an ascomycete, has been cited as the possible reason for the low transformation frequency for basidiomycetes including *Crinipellis perniciosa* (Lima *et al.*, 2003) and *Hebeloma cylindrosporum* (Marmeisse *et al.*, 1992). However, Tanguay et al. (2003) reported that although plasmid gGFP shared the same promoter region of P*gpd* gene from *A. nidulans*, PEG-mediated transformation frequency (based on hygromycin resistance) for gGFP was 2 transformants/μg of DNA per 10^8 protoplasts in the ascomycete

pathogenic fungus *Neonectria galligena* (Tanguay *et al.*, 2003). However, when they used the pAN7-1 vector to transform the same isolate, the transformation frequency was 30-60 times higher than for gGFP. This difference of transformation frequency was explained by the smaller size of pAN7-1 compared with gGFP (6.5 and 9.4 kb, respectively). The size of the plasmid pGpdGFP used in this study is 6.93 kb, approximately similar to pAN7-1. One can not rule out the possibility that the *trpC* promoter of *A. nidulans* in the plasmid pGpdGFP is poorly recognised by *T. basicola* giving a low transformation frequency of 2.5 transformants per μg of DNA. However, the bacterial *hph* gene under the control of the *A. nidulans trpC* promoter in pAN7-1 as a selectable marker generated 10^4 *Ophiostoma piceae* and *O. quercus* transformants per μg of DNA (Wang *et al.*, 1999). It is possible that the difference in the recognition of promoters could be explained by certain biological differences between the fungal species tested (Fincham, 1989).

Strategies for improving transformation efficiency of stable transformants include the addition of heat-denatured lambda DNA or a restriction enzyme in the transformation mixture. This approach has been reported to increase the transformation frequency of the basidiomycete *Pleurotus ostreatus* (Irie *et al.*, 2001) and the ascomycetes, *M. grisea* (Sweigard *et al.*, 1998) and *A. nidulans* (Karos and Fischer, 1996; Sanchez *et al.*, 1998).

Contaminating nucleases (highly active DNases secreted by fungi) have been reported to decrease the transformation frequency of stable transformants. Some species of fungi, for example, *Rhizoctonia solani* are presumed to secrete highly active DNases and the incorporation a DNase inhibitor in addition to an increase in plasmid concentration enabled successful transformation (Robinson and Deacon, 2001). However, all these options have yet to be tested on *T. basicola.*

The current establishment of the PEG-mediated transformation protocol for *T. basicola* will allow the screening of mitotically stable putative transformants to identify genes predicted to be involved in the pathogenicity of this organism.

3.5. Summary

On the basis of the results presented in this Chapter, the following protocol appears to be optimal for the production and transformation of protoplasts from endoconidia of *T. basicola* using the PEG/calcium chloride method.

A. Preparation of protoplasts

1. Using a sterile scraper, gently scrape spores of 3 day old plates (15-20 plates) of *T.basicola* (cotton isolate BRIP40192) grown on ½ PDA (2.2% agar). Dispense the scraped spores into 15 mL Tween 80 solution and vigorously vortex this suspension for 1 min.

2. Using a dried Mira cloth, (sterilized overnight by soaking it in 70% ethanol and dry for 30 min before filtration) separate the chlamydospores and mycelia from endoconidia by collecting the filtrate into 200 mL of PDB (1 liter flask).

3. Incubate the 200 mL endoconidial suspension at 25°C at 120 rpm for 2 h.

4. Dispense the mycelial suspension in sterile Beckmen centrifuge tubes (40 mL) and centrifuge at 2500 × *g*, 5 min at 4°C. Pour off the supernatant without disturbing the pellet.

5. Resuspend the pellet gently in 10 mL of ice-cold 0.6 M $MgSO4.\ 7H_2O$ and centrifuge at 2500 × *g*, 5 min at 4°C.

6. Pour off the supernatant and resuspend the pellet again in 10 mL of ice-cold 0.6 M $MgSO4{\cdot}7H_2O$. Centrifuge at 2500 × *g*, 5 min at 4°C. Repeat again.

7. Discard supernatant and suck off any extra liquid using a sterile pipette and record wet weight of pellet.

8. Keep wet mycelial pellet on ice and weigh Glucanex enzyme 5 mg/mL in ice cold osmotic media and vortex to resuspend. Use pipette to break up clumps of pellet and transfer the spore suspension into a sterile 50 mL flask. Add the enzyme to the spore suspension.

Mycelial weight (gram)	Enzyme in Osmotic medium solution 5 mg/mL
0.1-0.25 g	25 mg/5 mL
0.3-0.50 g	50 mg/10 mL
0.6-1.0 g	75 mg/15 mL

9. Gently swirl the flask so as to mix the contents and incubate at 30°C 120 rpm for 2 h.

10. Cool the flask on ice and swirl vigorously to free protoplasts from the mycelial debris. At this stage protoplasts can be observed under the light microscope.

B. Harvesting protoplasts (protoplasts should be kept on ice at all times)

1. Dispense the contents of the 100 mL flask into equal amounts in 2 sterile Corex tubes. Rinse the flask with 4 mL osmotic medium and add an equal volume to each Corex tube.

2. Gently overlay each Corex tube with 5 mL of trapping buffer. Centrifuge at 4000 × *g* in Beckman centrifuge for 20 min at 4°C.

3. To pellet and wash protoplasts, remove protoplast band from both Corex tubes using a sterile Pasteur pipette (heat in the Bunsen burner flame at right angle 1 cm from the tip) and dispense into one sterile Corex tube

4. Add 10 mL of ice-cold 1× STC to the protoplasts and gently resuspend six times. Centrifuge at 5000 × *g* for 5 min at 4°C.

5. Gently pour off the resultant supernatant and wash the pellet (protoplasts) again by gently resuspending in 10 mL of ice-cold 1× STC six times. Centrifuge at 5000 × *g* for 5 min at 4°C.

6. Repeat the washing procedure.

7. Gently resuspend the protoplast pellet in 4 mL ice cold 1× STC and count the protoplasts.

8. Aliquot in 150 µL lots in 2 mL Microcentrifuge tubes.

C. Transformation of *Thielaviopsis basicola* (BRIP40192) with pGpdGFP 6.93 kb (Selectable marker, hygromycin B).

Aliquot	Protoplasts (2 ×10^6) in 1× STC (µL)	+/-DNA (10 ug)	2× STC (µL)	1× STC (µL)	PEG (1st Incub. Temp.) (µL)	PEG (2nd Incub. Temp.) (mL)
1	150	18.2 µL	18.2	13.6	100	1
2(Control)	200	None	None		100	1

1. Add 100 µL of PEG in aliquots 1 and 2. Mix gently by tapping tubes several times and incubate on ice for 20 min.

2. Add 1 mL of PEG in aliquots 1 and 2. Mix gently and incubate at room temperature for 15 min.

3. Remove PEG by gently inverting to mix and centrifuge at 20,000 × *g* (14,000 rpm (bench top centrifuge) for 5 minutes. Tip off supernatant. Suck off any remaining PEG.

4. After removing PEG, resuspend and wash protoplasts in 1 mL ice cold 1× STC and centrifuge at 20,000 × *g* (14,000 rpm) for 5 min. Tip off supernatant. Suck off any remaining 1× STC. Tiny pellet can be seen. Repeat washing step again.

5. Gently resuspend all treatments in 200 µL ice cold 1× STC and then in 1.3 mL of PDB 0.5 M sucrose. Incubate with shaking at 120 rpm for 4 h at 25°C

6. After 4 h incubation, plate 50 µL on ½ PDA (1.2% agar) supplemented with 25 µg/mL of hygromycin B. Incubate all plates for 2 weeks at 25°C.

3.6. Conclusion

A protocol for the transformation of the filamentous fungus *T. basicola* was developed using the PEG/$CaCl_2$ method. Protoplasts were produced by digesting germinating endoconidia in a Glucanex solution osmotically stabilized with 1.2 M $MgSO_4$. The protoplasts were transformed with the plasmid pGpdGFP containing the bacterial hygromycin phosphotransferase gene (*hph*) conferring resistance to hygromycin B and incubated in regeneration broth for 4 h before being plated onto ½ PDA with 1.2% agar containing hygromycin B. Transformation frequencies of 2.5 hygromycin B-resistant transformants/µg of transforming DNA in treatments containing 2×10^6 protoplasts were obtained. Southern analysis of genomic DNA from the putative transformants, separated by agarose gel electrophoresis, confirmed that hygromycin B resistance resulted from integration of the plasmid pGpdGFP. Mitotic stability analysis revealed that 90.5% of transformants resistant to 100 µg/mL hygromycin B were mitotically stable.

Chapter 4 . Screening of *T. basicola* transformants and phenotypic and molecular characterisation of pathogenicity mutants

4.1. Introduction

The objective of random insertional mutagenesis in this project was to create transformants with single plasmid integration events at different locations in the fungal genome to identify genes involved in pathogenicity. Therefore, achieving this aim was highly dependent on designing a screening procedure that would easily identify the desired phenotype. The screening system had to be rapid, sensitive and reproducible. Screening for transformants with altered or reduced pathogenicity involved inoculating spores onto host plants and comparing the level of disease with plants inoculated with the wild-type. In some pathogenic fungi, it is possible to screen mutants for loss of a phenotype associated with pathogenicity using *in vitro* tests. For example, Lu *et al.* (1994) used a microbial assay to screen 1310 *Cochliobolus heterostrophus* transformants for T-toxin production. However, in the case of *T. basicola*, there was insufficient knowledge about pathogenicity factors to allow such an approach.

Random insertional mutagenesis has led to the discovery of many fungal pathogenicity genes (Xu *et al.*, 2006). Phenotypic and molecular characterisation of mutants has the enormous potential of providing us with exquisite knowledge of plant infection and colonisation by fungal pathogens.

Despite the well documented infection process of *T. basicola*, the molecular basis of pathogenicity is still unknown. To advance our understanding of disease mechanisms, pathogenicity genes can be identified by screening transformants for reduced or impaired pathogenicity.

In this study, 202 hygromycin-resistant *T. basicola* transformants were screened for altered pathogenicity and directional growth towards cotton using two rapid and reliable bioassays, the dipping technique and a water agar bioassay, respectively, already described in Chapter 2 of this thesis. Reduced pathogenicity of mutants obtained was further confirmed using an *in vitro* soil bioassay. Southern hybridisation analysis was used to confirm the integration of the transforming plasmid DNA. Attempts were made to identify the disrupted genes using plasmid rescue but were incomplete due to time limitation. The pathogenicity mutants were further characterised for pathogenicity towards lupin seedlings, germination, growth rate, sporulation, melanin production, adaptation to osmotic stress and lesion development 24 h after inoculation, in order to gain an insight on the effects of the mutation on the fungal phenotype.

4.2. Materials and Methods

4.2.1. Culture conditions for *T. basicola*

Stock cultures of *T. basicola* transformants obtained from the isolate BRIP40192 were maintained on agar blocks in sterile distilled water at room temperature or in 10% glycerol at -70°C. Working cultures were maintained as colonies on ½ PDA (2.2% agar) (section 2.2.2.1) and stored at 4°C for six months.

4.2.2. Sterilization of cotton and lupin seeds

Cotton and lupin seeds were sterilized and germinated as described in section 2.2.4.

4.2.3. Inoculum production of *T. basicola* transformants for pathogenicity and directional growth tests.

For primary screening assays the spore suspension used as an inoculum was not filtered and contained mycelia, chlamydospores and endoconidia. Spore suspensions were prepared by scraping 5 days old cultures of *T. basicola* wild-type or transformants grown on ½ PDA (2.2% agar) plates under a 12 h light/12 h dark cycle, into sterile McCartney bottles containing 10 mL of deionised water and vortexing the spore suspension at high speed for 1 min. A volume of 70 µL of the spore suspension was used as an inoculum for directional growth tests. A volume of 5 mL of the same spore suspension was used as an inoculum for pathogenicity tests.

4.2.3.1. Pathogenicity test on cotton roots using the dipping technique

For the identification of mutants showing reduced pathogenicity towards cotton, *T. basicola* transformants were subjected to the dipping technique as described in section 2.2.5 with the exception that the spore suspension contained mycelia, chlamydospores and endoconidia. Each experiment was conducted once with five replicate seedlings for each

treatment. Transformants that exhibited reduced pathogenicity compared to the wild-type were further analysed (section 4.2.4).

4.2.3.2. Growth of *T. basicola* transformants towards cotton roots

For the identification of mutants showing changes in hyphal growth towards cotton roots, *T. basicola* transformants were subjected to directional growth tests as described in section 2.2.7 with the exception that the spore suspension contained mycelia, chlamydospores and endoconidia. The experiments were conducted once with two replicates for each treatment.

4.2.4. Confirmation of pathogenicity and directional growth of *T. basicola* pathogenicity mutants

4.2.4.1. Inoculum production for *T. basicola* pathogenicity mutants

For further confirmation and characterization of *T. basicola* pathogenicity mutants, endoconidia were used as an inoculum. Endoconidial suspensions were prepared as described in section 2.2.3.

4.2.4.2 Pathogenicity of *T. basicola* mutants on cotton and lupin roots

For further confirmation of the validity of *T. basicola* mutants demonstrating reduced lesions compared to the wild-type, they were further subjected to the dipping technique, this time using known concentrations of endoconidia as inoculum rather than a spore suspension containing mycelia, chlamydospores and endoconidia. The dipping technique was performed as described in section 2.2.5. Seven days after inoculation, roots were examined for lesions as described in 2.2.5 and recorded from 5 replicate seedlings for wild-type *T. basicola* or each mutant for each inoculum concentration of 3.5×10^5 endoconidia/mL, 3.5×10^4 endoconidia/mL or 3.5×10^3 endoconidia/mL. Experiments were conducted three times.

Because most of the *T. basicola* isolates tested in Figure 2.2. were found to be highly pathogenic to lupin seedlings, causing 80-90% necrotic lesions, the pathogenicity of *T. basicola* mutants on lupin seedlings was tested using the dipping technique as described in section 2.2.5, but using an inoculum concentration of 3.5×10^4 endoconidia/mL. Seven days after inoculation, roots were examined for lesions as described in section 2.2.5 and recorded from 5 replicate seedlings for wild-type *T. basicola* or each mutant. The experiment was conducted once.

4.2.4.3. Pathogenicity of *T. basicola* mutants on cotton roots using *in vitro* soil bioassays

For further confirmation of reduced pathogenicity demonstrated by *T. basicola* mutants, mutants or wild-type (positive control) were subjected to an *in vitro* soil bioassay. The soil used for pathogenicity tests consisted of 50% black cracking clay (from Narrabri, NSW) and 50% sand thoroughly mixed and sterilised 3 times by autoclaving for 20 min (Mijajlovic, 2005). One litre of sterile soil was inoculated with 5 mL of 1×10^6 endoconidia/mL of *T. basiocla* mutants to achieve a final concentration of 2500 endoconidia/g of soil (5000 endoconidia/mL of soil). The endoconidial soil mixture was thoroughly mixed to ensure uniform dispersion of endoconidia throughout the soil. Negative controls consisted of uninoculated soil supplemented with a volume of 5 mL of sterile de-ionised water. A volume of 100 mL inoculated soil or control soil were then transferred to 175 mL foam cups and treated with 10 mL sterile de-ionized water. A surface sterilized cotton seed was placed in each cup and incubated at 25°C under a 12 h light/12 h dark cycle for 10 days and watered every 2 days with 10 mL sterile de-ionised water. Prior to examination, seedlings were carefully removed from the soil and washed with sterile de-ionised water. Disease severity was assessed as in section 2.2.5 and recorded from 10 replicate roots for each wild-type *T. basicola* or each mutant. Each experiment was conducted three times.

4.2.4.4. Directional growth of *T. basicola* mutants towards cotton roots

In order to confirm spore germination and growth in response to cotton roots, *T. basicola* mutants that showed reduced pathogenicity were subjected to a directional growth bioassay starting from endoconidia rather than mycelia. Directional growth assays were conducted using 1.2% Noble agar (2.2.2.3) on round plates (90 ×14 mm, vented) as described in section 2.2.7. The experiment was performed one time with five replicates of each *T. basicola* mutant or wild-type.

4.2.5. Molecular analysis of *T. basicola* pathogenicity mutants

The insertion of the plasmid pGpdGFP into the fungal genome of the pathogenicity mutants was confirmed by Southern hybridisation analysis. Genomic DNA was extracted as described in section 3.2.9.1 and digested with *Nhe*I, *Hpa*I, *Nru*I or *Xba*I (Genesearch New England Biolabs) under conditions specified by the manufacturer. *Nhe*I, *Hpa*I and *Nru*I do not cut within pGpdGFP, whereas *Xba*I has a unique site in the plasmid. The digests were allowed to incubate and after 18 h the reaction was stopped with 2 µl gel-loading dye (Table 3.1). The digests were separated in agarose gels as described in section 3.2.9.3 and blotted to positively charged nylon membrane as described in section 3.2.9.2. The Southern blot membranes were probed as described in section 3.2.9.2. The migration of each hybridizing band away from the well was measured in cm using a ruler. The sizes of the Lambda *Hin*d III standards and hybridizing bands were then determined using Sequaid software (Schaffer and Sederoff, 1981).

4.2.6. Characterisation of *T. basicola* pathogenicity mutants

4.2.6.1. Morphology of chlamydospores

Spores were scraped from 12 days old cultures of *T. basicola* mutants or wild-type and chlamydospores were examined microscopically for prominent changes in their morphological appearance compared to the wild-type.

4.2.6.2. Melanin production

Endoconidial suspensions were prepared by scraping 5 days old cultures of *T. basicola* wild-type or mutants grown on ½ PDA (2.2% agar) at 25°C under a 12 h light/12 h dark cycle, into sterile McCartney bottles containing 5 mL of deionised water and vortexing the spore suspension at high speed for 1 min. The spore suspensions were filtered through one layer of dry Miracloth (soaked overnight in 70% ethanol) to separate chlamydospores and mycelia from endoconidia. A volume of 200 µL uncounted endoconidial suspensions were grown in 30 mL PDB at 25°C under a 12 h light/12 h dark cycle at 120 rpm and flasks containing these cultures were visually examined and photographed after 7 days for melanin production.

4.2.6.3. Vegetative growth rate and spore count

The vegetative growth rate on ½ PDA (2.2%) and 1.2% water agar was conducted and determined as described in section 2.2.6. The experiment was conducted three times with three replicates for each treatment.

For chlamydospore counts, 12 days old cultures grown on ½ PDA (2.2%) plates which were incubated at 25°C under a 12 h light/12 h dark cycle were used. A drop of 50% ethanol was placed on each plate half way along the colony radius from the center to wet the mycelium and a coverslip was placed on the drop of ethanol. Chlamydospore chains from each replicate were observed under the microscope and counted in two fields of

0.0625 mm^2 with a 40× objective and the average determined. The experiment was performed once with three replicates for each treatment.

For endoconidia counts, endoconidial suspensions were prepared from the 12 days old plates by scraping cultures of *T. basicola* wild-type or *T. basicola* pathogenicity mutants into sterile McCartney bottles containing 7 mL of deionised water and vortexing the spore suspension at high speed for 1 min. The spore suspensions were filtered through one layer of dry Miracloth (soaked overnight in 70% ethanol) to separate chlamydospores and mycelia from endoconidia. The number of endoconidia was estimated using a hemocytometer. The experiment was performed twice with three replicates for each treatment.

4.2.6.4. Adaptation to osmotic stress

The effect of osmotic stress on vegetative growth rate of *T. basicola* mutants or wild-type was conducted on ½ PDA (2.2%) supplemented with 0.5%, 1.0% or 2% sodium chloride. Growth rates were determined as described in section 2.2.6. Controls did not include the supplementation of sodium chloride. The colony diameter was measured after 7 days. The experiment was conducted once with three replicates for each treatment.

4.2.6.5. Germination in liquid media

Germination of endoconidia was assessed in liquid Czapek Dox medium or PDB. Czapek Dox was used because it is a defined medium and PDB is a natural medium. The germination experiment was performed by dispensing 200-400 µL aliquots of 10^5 endoconidia/mL in 50 mL flasks containing 5 mL of PDB or liquid Czapek Dox, respectively. Flasks were incubated at 25°C in the presence of light at 120 rpm. At least 100 endoconidia in each replicate were examined microscopically for germination after 2 h incubation. The experiment was conducted once with three independent replicates for each strain and treatment.

4.2.6.6. Lesion development after 24 h post-inoculation

Two days old germinating cotton seedlings with radicles of uniform length (1 cm) free from microbial contamination were collected and placed on 0.8% water agar plates (90 ×14 mm, vented) containing 100 µL of 3.5 × 10^5 endoconidia/mL of *T. basicola* mutants or wild-type spread uniformly. Control seedlings were placed on 0.8% water agar plates not containing endoconidia. All plates were incubated at 25°C under a 12 h light/12 h dark cycle. Roots were examined for lesions under the stereo microscope 24 h post-inoculation and photographed.

4.2.7. Statistical analysis

The data obtained for pathogenicity tests on water agar plates and in soil, directional growth, growth rate, spore count were analysed by ANOVA as in section 2.2.8. Data for percent germination of endoconidia were arcsine-transformed prior to ANOVA.

4.2.8. Plasmid rescue

Attempts to rescue the regions flanking the inserted DNA in the *T. basicola* pathogenicity mutants were performed. Genomic DNA (400 ng or 1 µg) of mutants P16, P849 and P954 were digested with *Hpa*1 or *Nru*1. Mutant P849 was also digested with *Nhe*I. *Hpa*I, *Nru*I and *Nhe*I do not cut the transforming plasmid but cut within the flanking genomic DNA. Genomic DNA of all mutants was also digested with *Xba*I which cuts the plasmid once in a region not required for replication in *E. coli*. The digested-genomic DNA was allowed to incubate for 18 h at 37°C and was heat treated to inactivate the restriction enzymes according to the manufacturer's instructions. Five microliters of the digests were run on 0.8% agarose gel in 1× TEAC buffer (Table 3.1) at 60 volts for 3 h along side Lambda *Hind* III kb standards to confirm that the genomic DNA was completely digested. The digested-genomic DNA were then purified by extracting once with phenol-chloroform isoamyl alcohol (25:24:1 v/v/v) (Sigma), once with chloroform isoamyl alcohol (24:1 v/v),

precipitated with ethanol and pelleted. The pellets were washed twice with 70% ethanol, air dried for 30 min room temperature and finally resuspended in 10 µL of 1× T.E. buffer pH 8.0 (Table 3.1) and allowed to dissolve overnight at 4°C.

In another attempt to rescue the regions flanking the inserted DNA, approximately 3 µg of genomic DNA of P737, P849 and P954 were digested with *Xba*I and allowed to incubate and after 18 h, the reaction was stopped with 2 µL gel-loading dye (Table 3.1). Approximately 1 or 2 µg of digested-genomic DNA of P737, P849 and P954 were separated in an agarose gel as described in section 3.2.9.3. Part of the gel that contained approximately 1 µg of digested-genomic DNA of P737, P849 and P954 was blotted to positively charged nylon membrane as described in section 3.2.9.2. The Southern blot membranes were probed as described in section 3.2.9.2. The other part of the gel that contained 2 µg of the digested-genomic DNA of P737, P849 and P954 was stored at 4°C until the Southern blot membranes were probed and hybridizing bands determined. Hybridising bands corresponding to P737, P849 and P954 digested with *Xba*I were gel purified (Qiaquick Gel Extraction kit; Qiagen) according to the manufacturer's instructions and finally resuspended in 20 µL of 1× T.E. buffer pH 8.0. The digested-genomic DNA was then subjected to ligation.

The genomic DNA was recircularized in a 100 or 200 µL ligation reaction consisting of 2 units of T4 DNA ligase (New England Biolabs) and the manufacturer's buffer. The ligation reaction was performed at 16°C overnight and inactivated by incubating at 65°C for 20 min (according to manufacturer's instructions) and precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, washed with 70% ethanol, and air dried for 15 min at room temperature. The samples were dissolved in 5 µL of 1× T.E. buffer pH 8.0. For positive control, 100 ng of pGpdGFP was digested with *Xba*I for 18 h at 37°C and

recircularized in a 20 μL ligation reaction consisting of 1 unit of T4 DNA ligase (New England Biolabs) and the manufacturer's buffer.

1 or 2 μL of the ligation mix was transformed into electroporation *E.coli* DH5α competent cells by electroporation according to the manufacter's instructions (BIO-RAD). *E.coli* DH5α electro-competent cells were prepared according to the manufacturer's instructions (BIO-RAD). Ten pg of pGpdGFP (positive control) was transformed into electroporation *E.coli* DH5α competent cells by electroporation according to the manufacturer's instructions (BIO-RAD).

E.coli DH5α ampicillin resistant colonies were examined for their plasmid content using the Roche miniprep kit according to the maufacturer's instructions and the plasmids were stored at -20°C in 10 mM Tris-HCl pH 8.0. For the identification of the correct size fragment, several restriction enzymes were used to digest the plasmids. The products were separated on a 1% agarose gel and visualized under UV light after ethidium bromide staining.

Further attempts to optimise conditions for the rescue of the regions flanking the inserted DNA were not performed due to time limitation.

4.3. Results

4.3.1. Primary screening assays of *T. basicola* transformants

A total of 202 hygromycin-resistant putative mitotically stable transformants of *T. basicola* isolate BRIP40192 were screened for reduced pathogenicity and changes in growth directionality towards cotton roots. The primary screen using all fungal mycelia showed that none of the transformants demonstrated changes in growth towards cotton roots. All *T. basicola* transformants behaved in a similar fashion to the wild-type in their growth towards cotton seedlings. Five (P16, P737, P849, P888 and P954) out of 202 hygromycin resistant transformants, showed reduced pathogenicity compared with the wild-type.

4.3.2. Pathogenicity of *T. basicola* mutants on cotton roots using the dipping technique bioassay

Statistical analysis of the five mutants tested at an increasing range of inoculum levels from 3.5×10^3 to 3.5×10^5 endoconidia/mL, revealed a significant difference in aggressiveness compared with the wild-type (Figure 4.1). The necrotic lesions caused by *T. basicola* mutants or wild-type were reduced to about half as the inoculum levels of endoconidia decreased by 10 fold. For, example, at 3.5×10^5 endoconidia/mL, the disease severity for the wild-type was approximately 100%, at 3.5×10^4 endoconidia/mL, 55% and at 3.5×10^3 endoconidia/mL, 25%. The inoculum level of 3.5×10^4 endoconidia/mL allowed the best discrimination between the five mutants with the mutant P849 showing the least aggressiveness, and the mutants, P737 and P954 showing the most aggressiveness towards cotton roots (Figure 4.1B). Negative control treatments did not demonstrate any lesions (Figure 4.2).

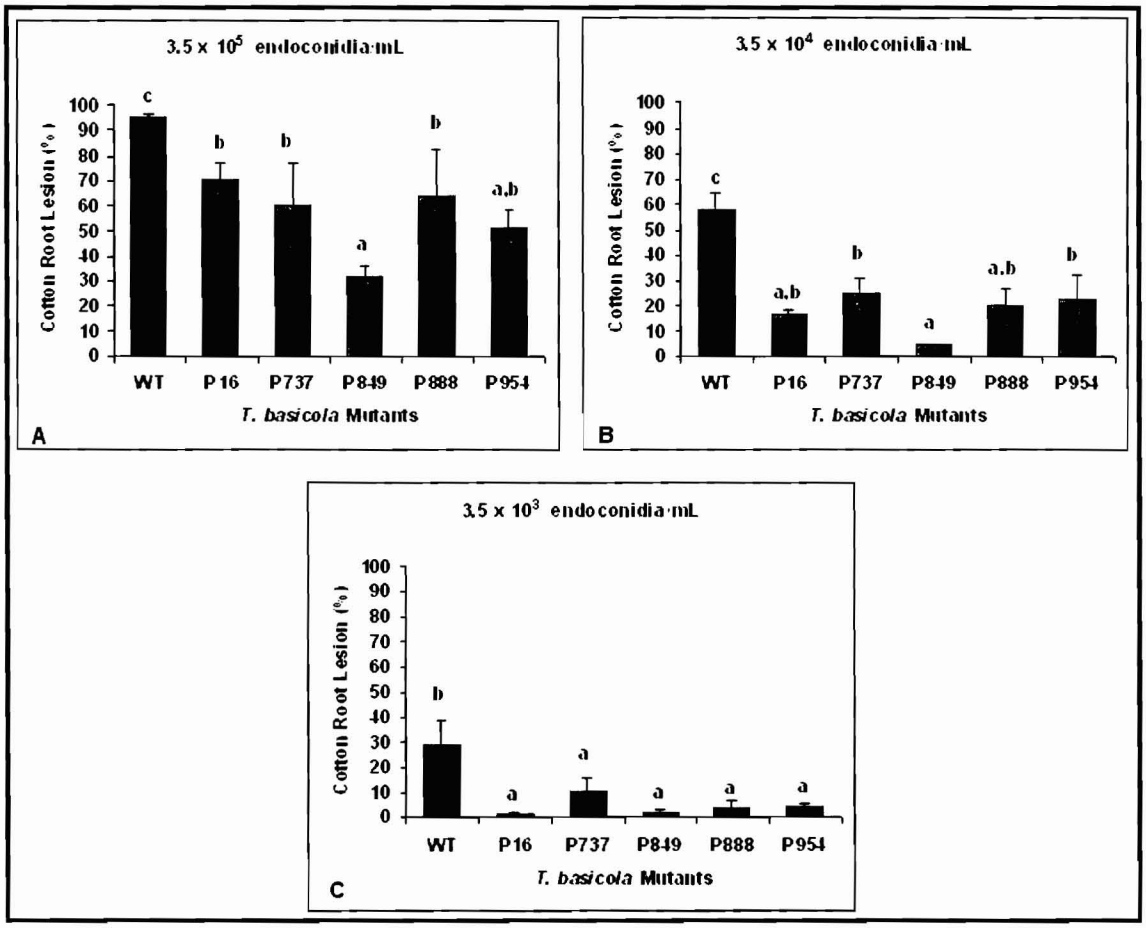

Figure 4.1. The effect of inoculum level on disease severity on cotton seedlings inoculated with *Thielaviopsis basicola* pathogenicity mutants using the dipping technique

Lesion percentage from inoculated roots with 3.5 × 10^5 endoconidia/mL (A), 3.5 × 10^4 endoconidia/mL (B) and 3.5 × 10^3 endoconidia/mL (C) was recorded 7 days post inoculation. Columns within concentrations labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical bars represent standard errors of the mean from three combined experiments with a total of 15 replicates for each inoculum level.

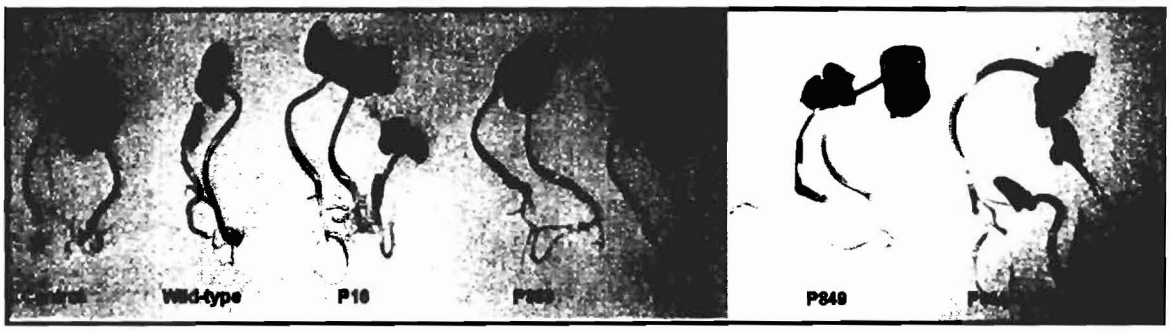

Figure 4.2. Representative roots of cotton seedlings exposed to *Thielaviopsis basicola* pathogenicity mutants

Pre-germinated cotton seedlings were inoculated by dipping in an endoconidial suspension (3.5 × 10^4 /mL) of each *T. basicola* mutant; P16, P888, P737, P849, P954 or *T. basicola* wild-type and incubated for 7 days at 25°C and then photographed. Control treatment consisted of dipping pre-germinated seedlings in sterile de-ionised water not containing endoconidia.

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Lupin roots subjected to 3.5 × 10^4 endoconidia/mL of *T. basicola* mutants showed significant reductions in necrotic lesions compared to lupin roots exposed to the wild-type 7 days post-inoculation. P954 was the most aggressive of the mutants causing 49% necrotic lesions on lupin roots. P16, P737, P849 and P888 showed necrotic lesions ranging from 4 to 21% (Figure 4.3). Lupin seedlings inoculated with de-ionised water not containing endoconidia showed no symptoms of disease (Figure 4.4).

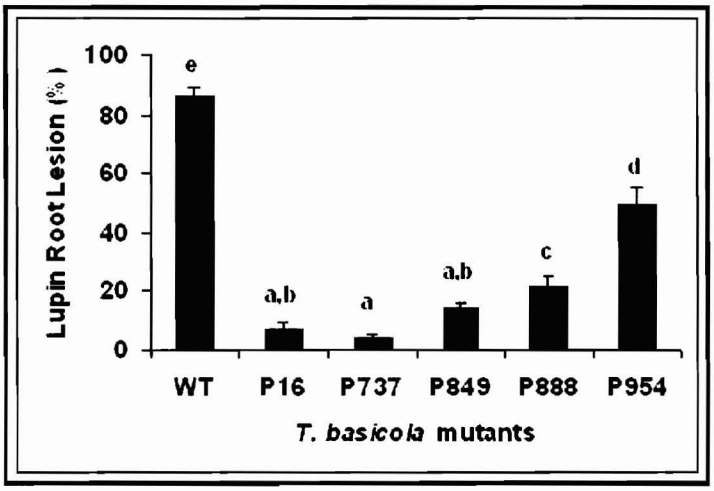

Figure 4.3. Disease severity of lupin seedlings inoculated with *Thielaviopsis basicola* pathogenicity mutants using the dipping technique

Lesion percentage from inoculated roots with 3.5 × 10^4 endoconidia/mL was recorded after 7 days post inoculation. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical bars represent standard errors of the mean from 8 replicates.

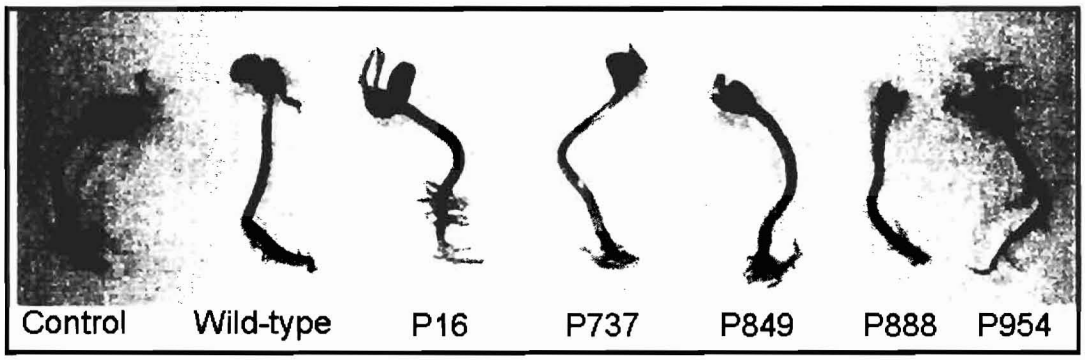

Figure 4.4. Representative roots of lupin seedlings infected with endoconidia of *Thielaviopsis basicola* pathogenicity mutants

Pre-germinated lupin seedlings were inoculated by dipping in an endoconidial suspension (3.5 × 10^4 /mL) of each *T. basicola* mutant; P16, P888, P737, P849, P954 or *T. basicola* wild-type and incubated for 7 days at 25°C and then photographed. Control treatment consisted of dipping pre-germinated seedlings in sterile de-ionised water containing no endoconidia.

4.3.3. Pathogenicity of *T. basicola* mutants on cotton roots using an *in vitro* soil bioassay

The five mutants were further tested for reduced pathogenicity in soil at an inoculum level of 2500 endoconidia/gram of soil. At this concentration of inoculum, the five mutants showed a significant decrease in pathogenicity in soil in comparison with the wild-type, with the least aggressive being P954 and the most aggressive being P16 and P888 (Figure 4.5). Negative control treatments did not show any root lesions (Figure 4.6).

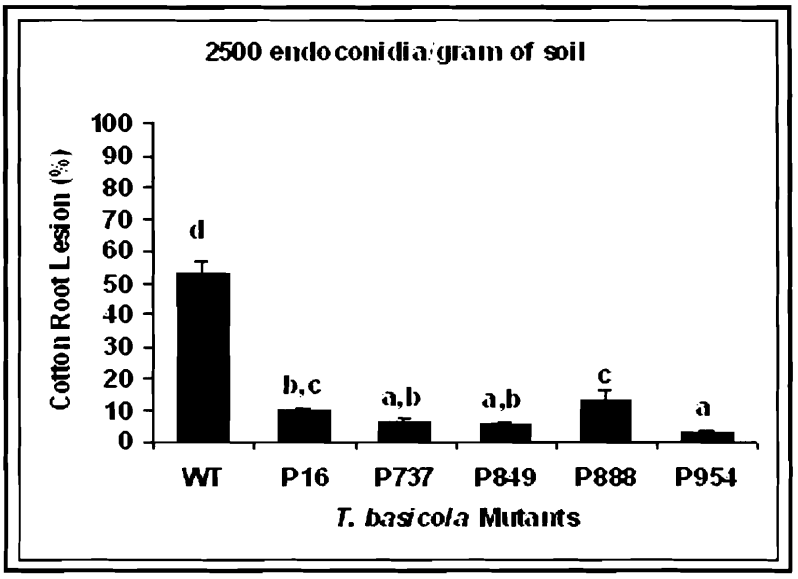

Figure 4.5. Disease severity on cotton seedlings in soil inoculated with *Thielaviopsis basicola* pathogenicity mutants

Lesion percentage from inoculated roots was recorded 10 days post-inoculation. Negative control treatment consisted of seeds grown in soil inoculated with de-ionised water. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical lines or bars represent standard errors of the mean from three combined experiments with a total of 30 replicates.

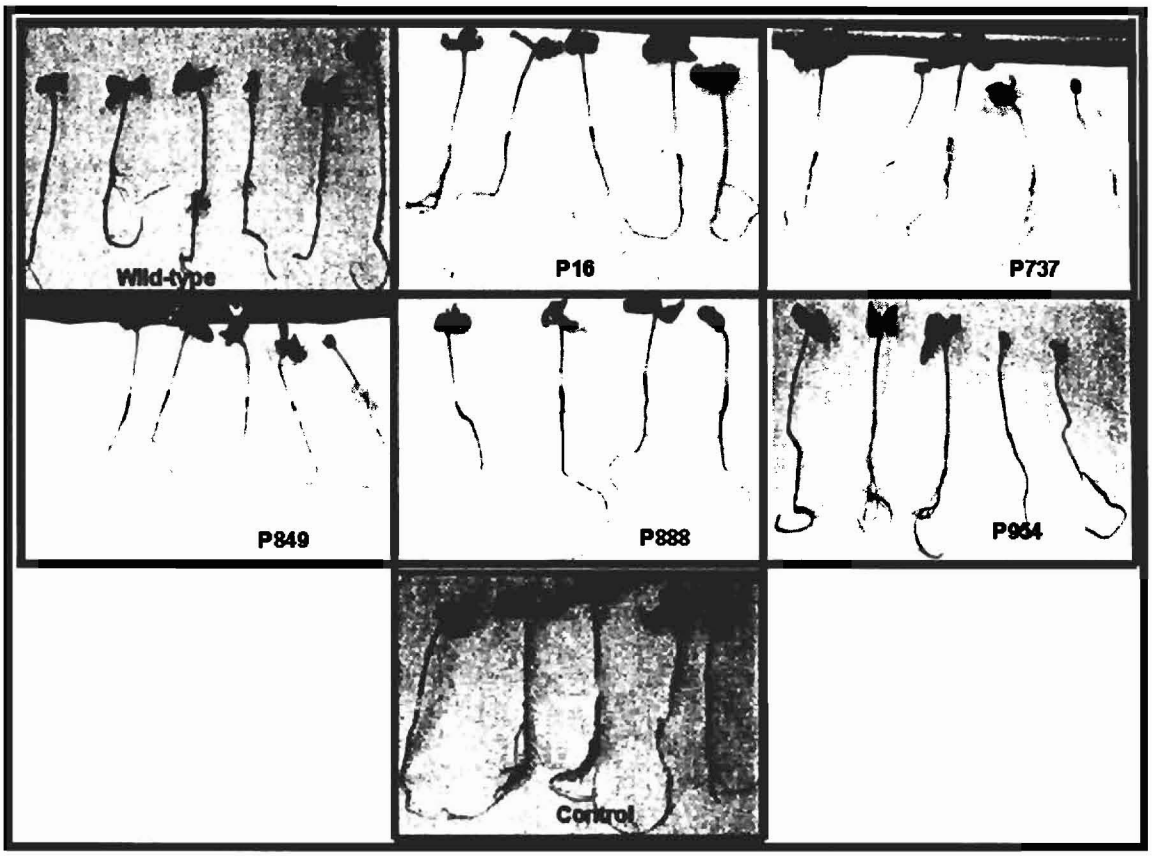

Figure 4.6. Representative roots of cotton seedlings grown in soil inoculated with *Thielaviopsis basicola* pathogenicity mutants

Cotton seeds were grown in inoculated soil (2500 endoconidia/gram of soil) of each *T. basicola* mutant; P16, P888, P737, P849, P954 or *T. basicola* wild-type and incubated for 10 days at 25°C and then photographed. Control treatment consisted of growing seedlings in soil inoculated with de-ionised water.

4.3.4. Growth of *T. basicola* mutants towards cotton roots

In order to confirm that pathogenicity mutants were able to germinate and grow towards cotton roots, pathogenicity mutants were subjected to directional growth tests using endoconidia as an inoculum instead of mycelia. The directional growth data for each *T. basicola* mutant and wild-type was adjusted according to their relative growth rates obtained on ½ PDA (2.2% agar) plates. The endoconidial germination and hyphal growth direction for P16, P737, P888 and P954 towards cotton roots was significantly higher than the wild-type. However, P849 was similar to the wild-type (Figure 4.7). Directional growth was not detected in negative controls.

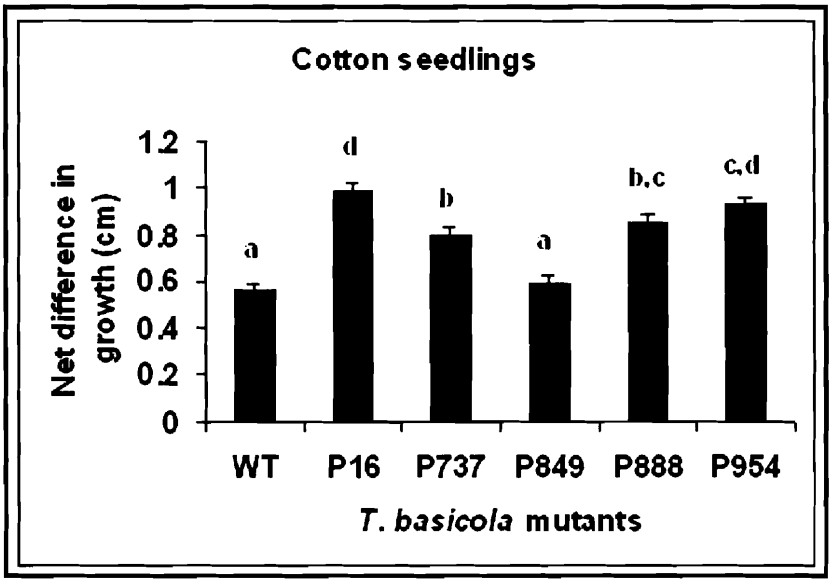

Figure 4.7. Growth of *Thielaviopsis basicola* pathogenicity mutants towards cotton seedlings

The data above shows net differences in growth towards and away from cotton seedlings adjusted for relative growth rates of *T. basicola* mutants and wild-type. Inoculum of 70 µL at a concentration of 3.5 $\times 10^4$ endoconidia/mL was applied to filter paper strips and exposed to 4 cotton seeds per plate. Growth of mycelia was measured after seven days post-inoculation. Columns labelled with the same letters do not differ significantly at $p < 0.0.5$ by Duncan's test. Vertical bars represent standard errors of the mean from 5 replicates.

4.3.5. Molecular analysis of *T. basicola* mutants

To confirm that *T. basicola* pathogenicity mutants contained integrated plasmid DNA, the mutants were subjected to molecular analyses. Genomic DNA from the five pathogenicity mutants digested with the enzymes, *Hpa*I, *Nhe*I, or with *Nru*I (which do not cut in pGpdGFP), showed hybridisation to fragments of different sizes using the pGpdGFP probe. The results presented in Figure 4.8 A and B, show that the plasmid pGpdGFP integrated into the genome of P16, P737, P849 and P954 at a single site giving a restriction fragment that was greater in size than the linearised plasmid (6.9 kb). P888 showed three hybridising bands when digested with *Nhe*I. One band represented a restriction fragment that was greater in size than the linearised plasmid (6.9 kb) and the other two bands represented restriction fragments smaller than the size of the linearised plasmid. The origin of these two bands was unclear. Digestion of P888 with *Hpa*I or *Nru*I revealed only one hybridizing band greater than the size of the linearised plasmid. All of the integration events resulted in approximately similar or different-sized but not in identical *Hpa*I, *Nhe*I or *Nru*I fragments, indicating that the plasmid pGpdGFP was possibly integrated at different chromosomal locations. P16 (Figure 4.8 A, lanes 2 & 8, Figure 4.8 B, lane 2) and P888 (Figure 4.8 A, lanes 5 & 11, Figure 4.8 B, lane 5) digested with any of these three enzymes, showed very strong hybridisation signals suggesting that tandem integrations of pGpdGFP may have occurred.

P737, P849 and P954 yielded weak hybridizing bands suggesting that not many copies of the plasmid had integrated. The sizes of the hybridizing bands were determined using Sequaid software (Schaffer and Sederoff, 1981). No hybridising bands were found in the genomic DNA of the recipient wild-type strain.

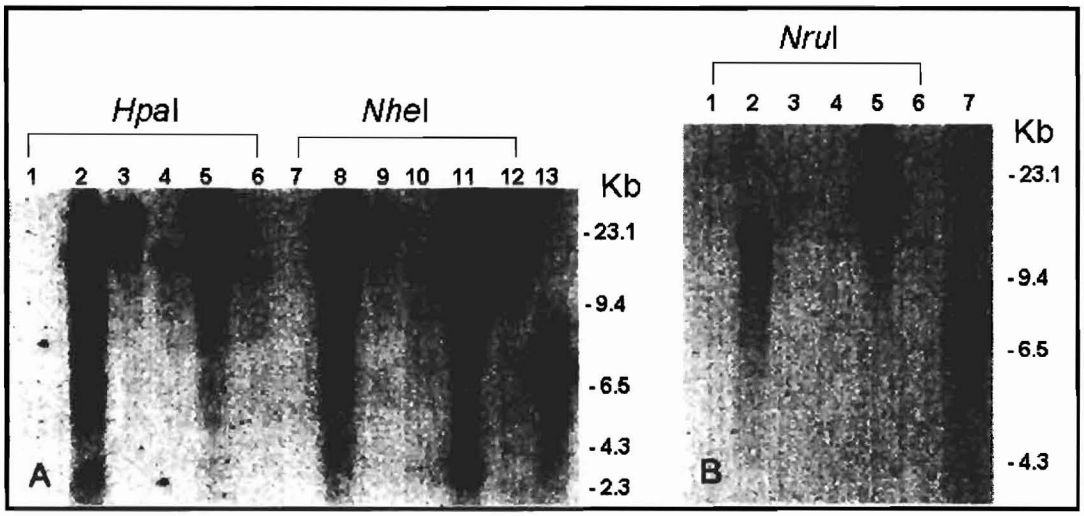

Figure 4.8. Southern hybridisation analysis of *Hpa*I-, *Nhe*I- or *Nru*I-digested genomic DNA of *Thielaviopsis basicola* pathogenicity mutants.

The genomic DNA was digested with *Hpa*I, *Nhe*I or *Nru*I which do not cut within the transforming plasmid pGpdGFP. (A) Lane 1 contains wild-type digested with *Hpa*I, lanes 2-6 contain the mutants: P16, P737, P849, P888, P954 digested with *Hpa*I, lane 7 contains wild-type digested with *Nhe*I, lanes 8-12 contain the mutants: P16, P737, P849, P888, P954 digested with *Nhe*I and lane 13 contains plasmid pGpdGFP linearised with *Xba*I (positive control).
(B) Lane 1 contains wild-type digested with *Nru*I, lanes 2-6 contain the mutants: P16, P737, P849, P888, P954 digested with *Nru*I, and lane 7 contains plasmid pGpdGFP linearised with *Xba*I (positive control).
Equivalent amounts of genomic DNA were loaded in all lanes and fractionated in 0.8% agarose gel at 38 V. Southern hybridisation analysis was carried out as described in section 3.2.9.2. Digoxigenin (DIG)-labeled plasmid pGpdGFP linearised with *Not*I was used as a probe. Some fragments of the molecular size marker Lambda (λ) *Hin*d III are indicated in kilobases on the right.

When the genomic DNA of the *T. basicola* pathogenicity mutants were cut with *Xba*I, which has one site in pGpdGFP, P737 (Figure 4.9 B, lane 3) and P888 (Figure 4.9 B lane 5) showed hybridizing bands corresponding to the molecular weight of the plasmid pGpdGFP (6.9 kb) (Figure 4.9 B, lane 1). P737 also showed two other additional hybridizing bands and P888, three more additional hybridizing bands the largest may indicate partial digestion of the genomic DNA. P16 (Figure 4.9 B, lane 2) showed a weak hybridisation band also corresponding to the molecular weight of the plasmid pGpdGFP. P16 also showed five more additional hybridizing bands. Each P849 and P954 showed three weakly hybridizing bands (Figure 4.9 B, lanes 4 and 6).This type of hybridisation pattern is not consistent with simple plasmid integration whereby a single integrated copy of the plasmid would produce two bands of a size other than 6.9 kb, reflecting the sequences flanking the plasmid.

These hybridisation patterns could also possibly indicate that P737, P888 and P16 contained more than one tandemly arranged integrated copy of the transforming plasmid DNA at a single locus. Strongly hybridizing bands observed in P16 and P888 not corresponding to fragments of the linearised plasmid DNA when compared to the size of the bands using *Hpa*I, *Nhe*I or *Nru*I (Figure 4.8 B) probably represent one or more copies of pGpdGFP sequences integrated in tandem.

The sizes of the hybridizing bands were determined using Sequaid software (Schaffer and Sederoff, 1981). No hybridising bands were found in the genomic DNA of the recipient wild-type strain.

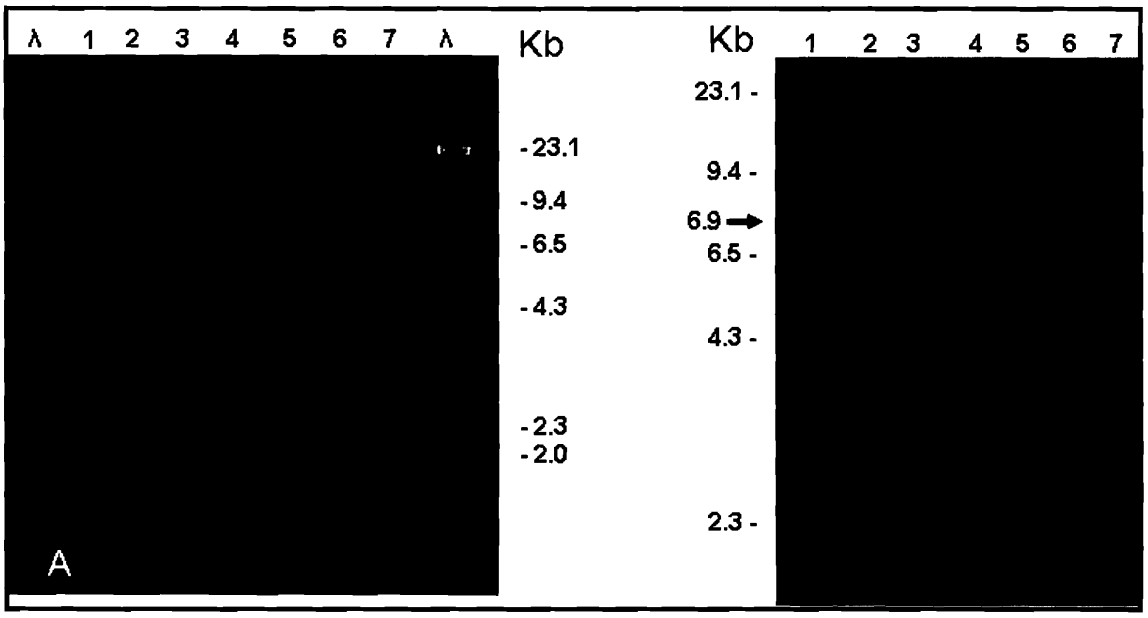

Figure 4.9. Southern hybridisation analysis of *Xba*I-digested genomic DNA of *Thielaviopsis basicola* pathogenicity mutants

The genomic DNA was digested with *Xba*I which cuts once within the transforming plasmid pGpdGFP. (A) Equivalent amounts of genomic DNA were loaded in all lanes and fractionated in 0.8% agarose gel at 38 V. Lane 1 contains pGpdGFP digested with *Xba*I (positive control), lanes 2-6 contain the mutants: P16, P737, P849, P888, P954 digested with *Xba*I, lane 7 contains wild-type digested with *Xba*I, λ represents Lambda *Hind*III used as a molecular size marker. Some fragments of the molecular size marker Lambda (λ) *Hin*d III are indicated in kilobases on the right.

(B) Southern hybridisation analysis was carried out as described in section 3.2.9.2. Digoxigenin (DIG)-labeled plasmid pGpdGFP linearised with *Not*I was used as a probe. Lane 1 contains pGpdGFP digested with *Xba*I (positive control) indicated by arrow, lanes 2-6 contain the mutants: P16, P737, P849, P888, P954 digested with *Xba*I, lane 7 contains wild-type digested with *Xba*I. Some fragments of the molecular size marker Lambda (λ) *Hin*d III are indicated in kilobases on the left.

4.3.6. Characterisation of *T. basicola* pathogenicity mutants

Alterations in other phenotypic characteristics were investigated in order to learn more about the effect of the mutation on the *T. basicola* pathogenicity mutants. The phenotypic characteristics studied included the morphology of chlamydospores, melanin production, vegetative growth rate, spore count, adaptation to osmotic stress and early stages of seedling infection.

4.3.6.1. Morphology of chlamydospore and melanin production

Light microscopy of mycelia of *T. basicola* mutants or wild-type from 12 days old cultures demonstrated morphological differences between the chlamydospores of the wild-type strain and P16. The majority of the wild-type chlamydospore chains consisted of 3-4 thick dark brown segments, with each segment containing an individual dark brown cylindrical cell (Figure 4.10 A) whereas in P16, the majority of chlamydospore chains were abnormal. They consisted of 5-7 light brown segments with each segment containing an individual light brown semi-cylindrical cell (Figure 4.10B). It was not investigated whether these segments separate into individual cylindrical cells, each of which may germinate, a characteristic reported for *T. basicola*. P849 and P954 also showed 3-4 thick segments but they were light brown.

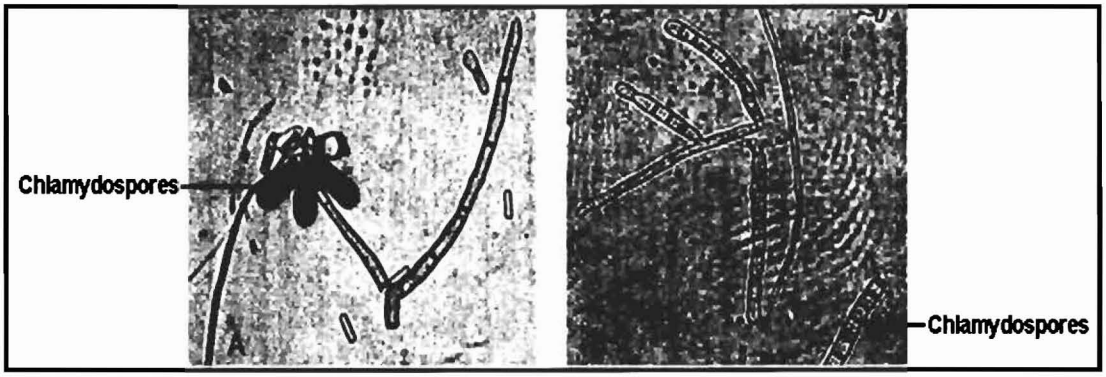

Figure 4.10. Chlamydospore morphology of P16

Light microscopy of chlamydospores of wild-type (A) and P16 (B) from 12 days old PDA culture plates. Chlamydospores are abnormal in the mutant P16.

From the five mutants that showed reduced pathogenicity, three (P16, P849 and P954) had reduced pigmentation of their chlamydospores and did not synthesize a dark brown pigment (melanin) when the mutants were grown in PDB. P737 and P888 synthesized chlamydospores that were similar in appearance to those of the wild-type, but had a darker brown pigment than the wild-type (Figure 4.11).

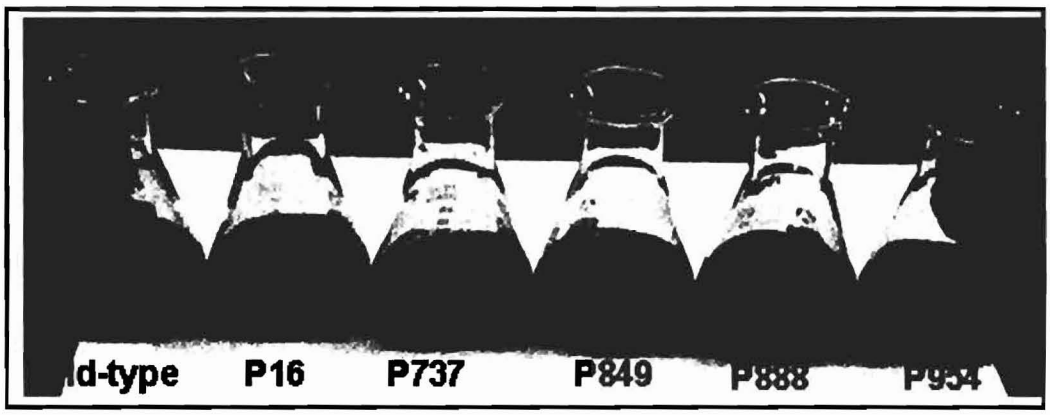

Figure 4.11. Appearance of *Thielaviopsis basicola* wild-type and pathogenicity mutants.

Flasks showing 7 days old cultures of *T. basicola* wild-type isolate BRIP40192 or mutants grown in 30 mL PDB. P16, P849 and P954 showed reduced melanin compared to *T. basicola* wild-type. P737 and P888 showed enhanced melanin.

4.3.6.2. Vegetative growth rate and spore count

Seven day growth rate (colony diameter) analysis of all mutants showed a slight but significant reduction compared to the *T. basicola* wild-type on PDA media (Figure 4.12 A) or water agar (Figure 4.12 B) except for P954 which was similar to the wild-type. P16 demonstrated the slowest growth on PDA and P737 was the slowest on water agar compared to the other mutants. Both mutants and wild-type grew slightly faster on PDA than on water agar.

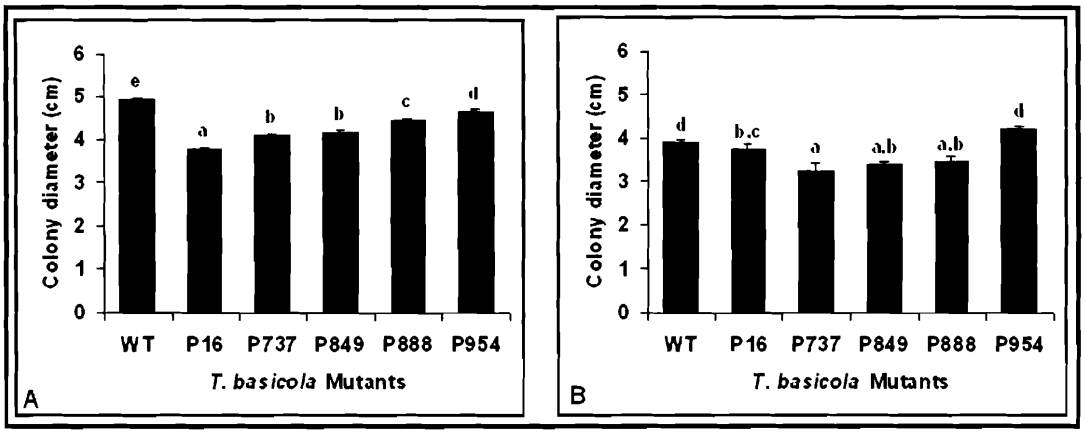

Figure 4.12. Assessment of vegetative growth of *Thielaviopsis basicola* pathogenicity mutants.

Wild-type (WT) *T. basicola* isolate BRIP40192 and mutants were grown on PDA (A) and water agar (B) at 25°C for 7 days and colony diameters were measured. Data represent the growth after 7 days. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical bars represent standard errors of the mean from three combined replicates.

After 12 days incubation at 25°C, P849 produced the highest number of endoconidia in comparison with the other mutants or wild-type, whereas, P16, P737, P888 and P954 produced similar number of endoconidia compared to the wild-type (Figure 4.13 A). P849 also demonstrated the highest number of chlamydospore chains/mm^2 compared to the other mutants or wild-type. P16 and P954 produced the lowest number of chlamydospores/mm^2 compared to other mutants or the wild-type. The number of chlamydospore chains/mm^2 produced in either P737 or P888 was not significantly different from the wild-type (Figure 4.13 B).

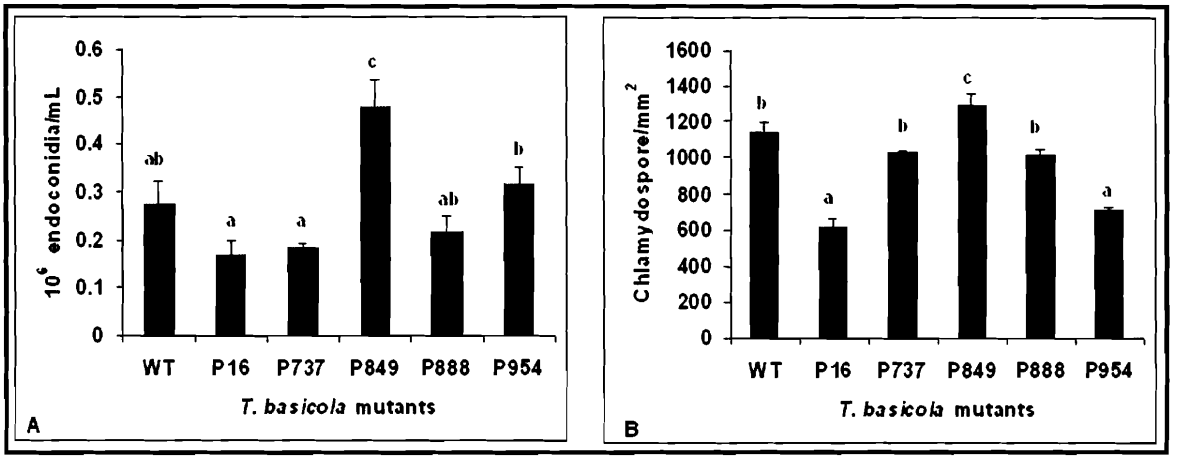

Figure 4.13. Assessment of spore count of *Thielaviopsis basicola* pathogenicity mutants on PDA.

Mutants and wild-type were grown on PDA for 12 days at 25°C Number of endoconidia (A) and chlamydospore chains (B) were counted as in materials and methods. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical bars represent standard errors of the mean from three combined replicates.

4.3.6.3. Effect of osmotic stress on vegetative growth rate

The response of mutants and wild-type *T. basicola* to osmotic stress is shown in Figure 4.14. All strains grew best on PDA without NaCl supplementation. Wild-type *T. basicola* showed significantly faster growth compared with the mutants followed by P954 and P888. P16 showed the slowest growth. The growth rate of wild-type *T. basicola* and mutants decreased as the concentration of NaCl increased (Figure 4.14). At 0.5% NaCl, P954 grew as fast as the wild-type, followed by P737, P849 and P888. P16 still showed the slowest growth amongst the mutants. The growth of P954 was faster than the wild-type at 1% NaCl. The growth rate of P16 at 1% NaCl was not significantly different from the wild-type. The colony diameters of P737, P849 and P888 were significantly lower from the corresponding wild-type in 1% NaCl. At 2% NaCl, all the strains showed a significant reduction in their growth rate and the mutants grew slower compared to the wild-type, except for P16 which was not significantly different from the wild-type.

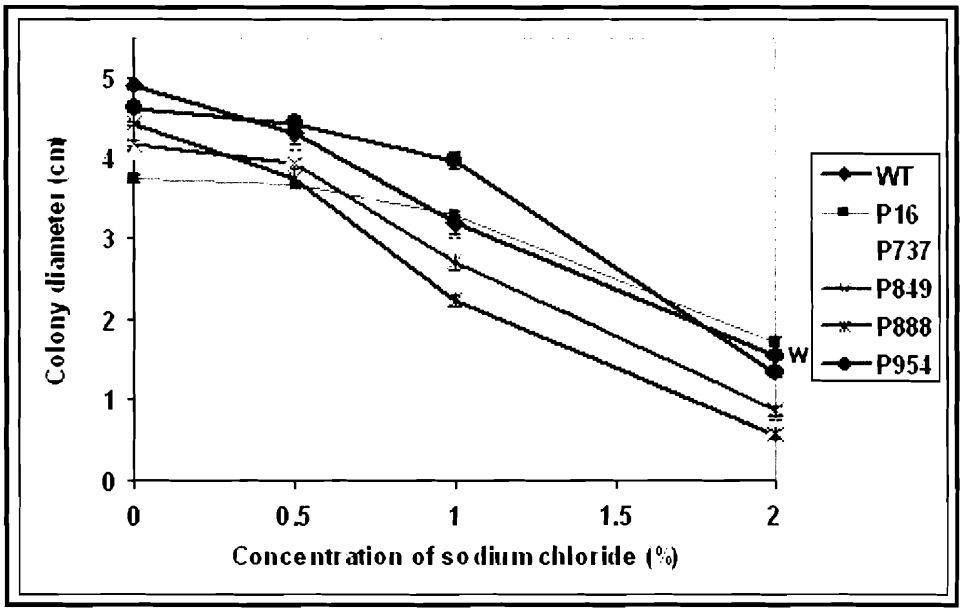

Figure 4.14. Effect of osmotic stress on vegetative growth of *Thielaviopsis basicola* pathogenicity mutants

Mutants and wild-type were grown on PDA amended with 0.5%, 1%, or 2% NaCl for 7 days and the colony diameter was measured. Controls lacked sodium chloride. Values are the means of 4 replicates. Vertical bars represent standard errors of the mean from three combined replicates.

4.3.6.4. Germination in liquid media

The germination rate of pathogenicity mutants after 2 h incubation in both PDB (natural medium) and Czapek Dox (defined medium) was either similar to or significantly higher than that of the wild-type (Figure 4.15). The germination percentage of wild-type was similar in PDB and Czapek Dox. The mutants; P16, P737, P849 and P888 demonstrated similar germination percentages when inoculated in PDB and Czapek Dox, except for P954, that showed the highest germination percentage in Czapek Dox compared to PDB.

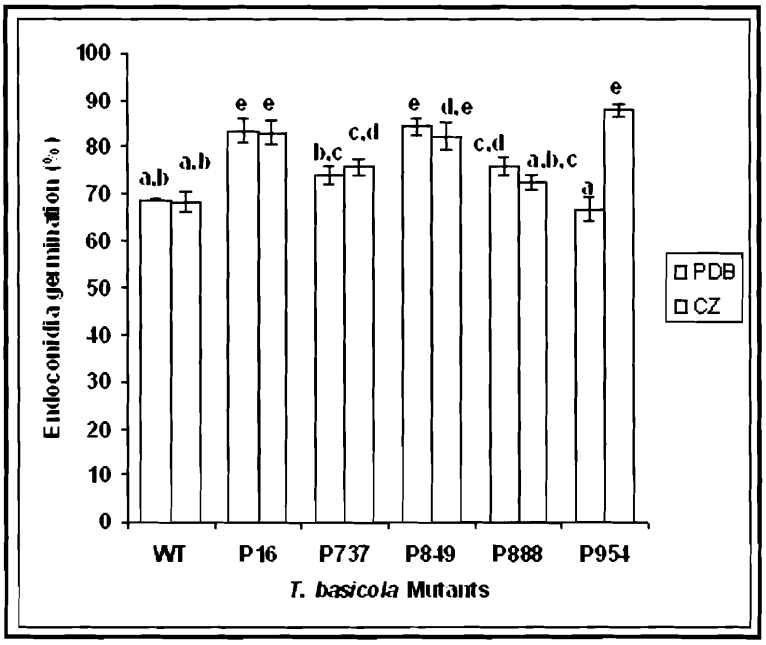

Figure 4.15. Germination of *Thielaviopsis basicola* pathogenicity mutants in liquid media

Germination was assessed following microscopic examination of 100 endoconidia per plate 2 h after application of 10^5 endoconidia/mL of each spore suspension into 5 mL of PDB with shaking at 120 rpm. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical bars represent standard errors of the mean from three combined replicates.

4.3.6.5. Lesion development 24 h post inoculation

Penetration and colonization of root hairs and epidermal cells was observed in all roots inoculated with pathogenicity mutants or wild-type *T. basicola* 24 h post-inoculation (Figure 4.16). Root hairs were absent in areas where brown lesions developed. Lesion development or colonization was not extensive and production of chlamydospores was not evident at this stage of the infection by either the wild-type or the mutants. No major differences were observed between all the strains in the colonization of cotton roots 24 h post-inoculation.

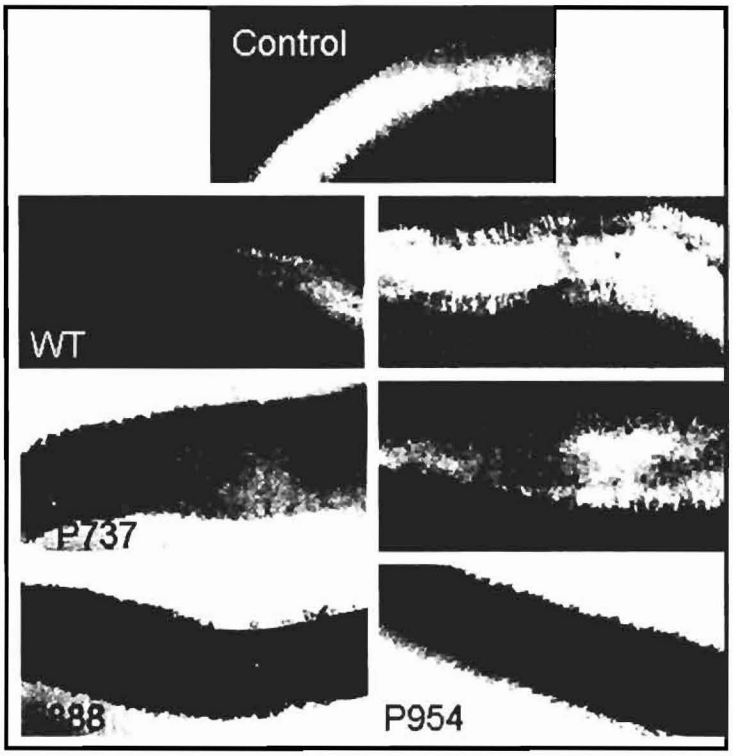

Figure 4.16. Colonisation of *Thielaviopsis basicola* pathogenicity mutants after 24 h post-inoculation

4.3.7. Plasmid Rescue

To obtain sequences flanking the plasmid, attempts to rescue the integrated plasmid containing the flanking genomic DNA were performed. Molecular analysis in section 4.3.5 indicated that P16, P849 and P954 digested with *Hpa*I or *Nru*I and P849 digested with *Nhe*I obtained size fragments ranging from 12–17 kb. These were selected for plasmid rescue analysis. Fragments larger than 17 kb were not subjected to plasmid rescue. Ampicillin resistant transformants of *E. coli* strain DH5α obtained, ranged from 2-10 colonies in each of several attempts. Ligated *Xba*I linearised plasmid (positive control) (10 pg) generated more than 500 ampicillin-resistant transformants of *E. coli* strain DH5α. Uncut plasmid DNA extracted from some of these transformants was identical or smaller than the uncut control transforming plasmid pGpdGFP (Figure 4.17). Restriction analysis with *Hpa*I, *Nru*I or *Nhe*I did not reveal the expected size fragments of 12-17 kb but

revealed complex restriction patterns (data not shown). Further restriction analysis with *Kpn*I or *Bam*HI (enzymes that cut twice within the plasmid) revealed size fragments similar to pGpdGFP in both molecular weight and restriction enzyme digestion pattern when compared to pGpdGFP cut with *Kpn*I or *Bam*HI (positive control) (data not shown).

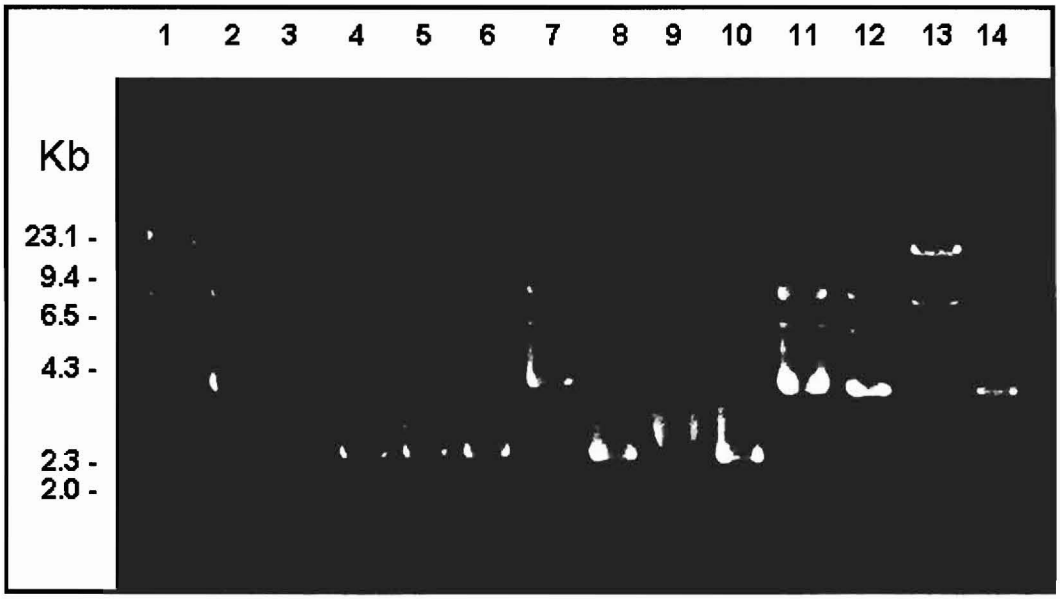

Figure 4.17. Mini prep products of uncut plasmid DNA from ampicillin-resistant transformants of *E. coli* strain DH5α.

Lane 1, Lambda (λ) *Hin*dIII markers Lane 2 *Nru*I-P954 fragment , lanes 3; *Hpa*I-P954 fragment, lanes 4-7, *Hpa*I-P16 fragment, lanes 8-10, *Nru*I-P16 fragment, Lane 11-12; *Hpa*I-P849 fragment, Lane 13; . Lane 1, Lambda (λ) *Hin*dIII markers, Lane 14: Ligated *Xba*I-pGpdGFP fragment. Lambda (λ) *Hin*dIII are indicated in kilobases on the left.

In order to further reduce the size of the restriction fragments generated from *Hpa*I-, *Nhe*I- or *Nru*I-digested genomic DNA, the genomic DNA was cut with *Xba*I that cuts within the plasmid DNA. This strategy would attempt to rescue the genomic DNA from only one side of the integration site which contains the ampicillin gene and the origin of replication. Mutants that were suspected to contain tandemly repeated copies of the plasmid were not subjected to plasmid rescue. P849 and P954 did not produce any *E. coli* resistant colonies when digested with *Xba*I. Ampicillin resistant transformants of *E. coli* strain DH5α obtained from *Xba*I-digested genomic DNA of P737 generated 2-5 colonies in each of several attempts. Undigested plasmid DNA observed for some of these transformants were similar to uncut pGpdGFP (data not shown). Restriction analysis was not performed.

Initial attempts to rescue the integrated plasmid derived from *Xba*I-digested genomic DNA fragments (not corresponding to the molecular weight of the plasmid pGpdGFP), that were subjected to a Southern hybridisation analysis and hybridizing bands excised and purified from a Southern blot gel, failed to retrieve any ampicillin resistant colonies. Further plasmid rescue analyses were not performed due to time limitation.

4.4. Discussion

In order to screen for reduced pathogenicity mutants of *T. basicola* on cotton seedlings, the dipping technique used in Chapter 2 of this thesis was adopted. The dipping technique was previously proven to be a simple, rapid and reliable pathogenicity test. Therefore, it was used as a primary bioassay to screen large numbers of transformants. Two days old cotton seedlings were used for screening as black root rot is a disease that attacks seedlings and, furthermore, older seedlings may be more resistant to infection probably due to formation of cork cambium (Mathre *et al.*, 1966).

Since soil pathogenicity assays are time and labour consuming, they were excluded from the primary screening and used only for mutant confirmation. Endoconidia are often used as a source of inoculum in phytopathological studies (Punja, 1993), however, isolating endoconidia from large numbers of transformants was also found to be time consuming. Therefore, a spore suspension containing mycelia, chlamydospores and endoconidia were used as a source of inoculum for the primary infection assay and endoconidia were used only in the confirmation stages.

The dipping technique had a disadvantage that it could not detect small differences in disease levels, therefore, only mutations that had a relatively large effect on pathogenicity were detected. The dipping technique also could not detect mutants that were defective in their ability to grow towards cotton seedlings or other susceptible plants. Therefore, directional growth assays (used in Chapter 2 of this thesis) were also applied here with the only difference that mycelia rather than endoconidia were used as an inoculum. None of the 202 transformants were found to be impaired in their ability to grow towards cotton roots including the pathogenicity mutants.

The five pathogenicity mutants reported here were isolated after screening 202 *T. basicola* transformants, corresponding to a frequency of 2.5%. This frequency is higher than reported by Baldahere *et al.* (1999) where they identified five pathogenicity mutants after screening 1150 *M. grisea* REMI transformants (0.4%) and also higher than Inoue *et al.* (2001) where they identified 43 mutants out of 2929 *F. oxysporum* REMI transformants (1.5%) and 2 out of 146 *F. oxysporum* PEG-mediated transformants (1.4%).

Once the five mutants were identified, their reduced pathogenicity was confirmed by conducting the infection and directional growth assay using endoconidia. A known source of inoculum was used in order to compare the level of disease between the mutants and the wild-type. Mathre *et al.* (1966) had previously shown that black root rot of cotton seedlings (Acala 4-42) can occur with inoculum densities as low as 100 endoconidia/g of soil. This study used 25 fold higher inoculum density of endoconidia/g of soil than that of Mathre *et al.* (1966) to test *T. basicola* pathogenicity mutants or wild-type allowing the mutants advantage over the plant.

Inoculation of young seedlings using an endoconidial inoculum concentration adjusted to 3.5×10^4 endoconidia/mL in the dipping technique provided the best discrimination between the wild-type and the mutants compared to the concentration inoculum of 3.5×10^5 endoconidia/mL or 3.5×10^3 endoconidia/mL. Therefore, this inoculum is recommended for a secondary screening for pathogenicity mutants on cotton seedlings.

Directional growth assays using a known concentration of inoculum of pathogenicity mutants confirmed the observations in the primary test which showed no defect in their capability to germinate and grow towards the cotton roots. Therefore, it seems that reduced pathogenicity in the mutants is linked to later stages in the infection process. Growth towards roots for the pathogenicity mutants was similar or significantly higher than the wild-type. Higher rates than the wild-type could be attributed to their faster germination

rate. All mutants did not demonstrate any delay in the onset of endoconidia germination and showed a higher germination rate after 2 h incubation in either PDB or Czapek Dox compared to the wild-type. But whether signals exuded from cotton roots play an important role in specifically triggering germination and directional attraction of these mutants remains to be investigated using other susceptible and non-susceptible hosts or non-host plants.

Both lupin and pansy seedlings were susceptible to most of the *T. basicola* isolates tested previously (Chapter 2). Only lupin seedlings were selected for further assessment of disease severity caused by the mutants, since they were easier to handle than pansy and reduced pathogenicity by the mutants was confirmed. P954 showed reduced pathogenicity towards cotton roots but as the wild-type, it was relatively virulent towards lupin seedlings. Evidence of such host specificity was also observed in the *T. basicola* mutant, P737. The *T. basicola* cotton isolate was highly pathogenic to cotton roots as well as lupin roots. However, P737 behaved differently from the wild-type towards cotton and lupin and may be affected in host-specificity related genes.

Southern hybridisation confirmed that the *T. basicola* pathogenicity mutants contained integrated transforming plasmid DNA. But whether the integrated plasmid DNA is responsible for the mutation that resulted in different phenotypic characteristics observed in these mutants needs to be confirmed. The confirmation process involves rescuing the integrated plasmid and flanking genomic DNA and then the rescued plasmid can be used to probe a Southern blot with DNA from the mutant and wild-type to verify that the plasmid did indeed identify a plasmid integration event (Sweigard, 1996). The sequences flanking the plasmid can be sequenced and the gene identified. To confirm that the retrieved genomic DNA contained sequences related to pathogenicity, the identified gene can be cloned and transformed into the wild-type *T. basicola* isolate BRIP40192 in an attempt to

disrupt the wild-type copy of the sequences responsible for the mutation and generate non-pathogenic mutants (Redman *et al.*, 1999). The mutation can then be complemented with a wild-type copy of the DNA, spanning the plasmid insertion site in order to confirm that the integration event is indeed responsible for the altered pathogenicity of the mutant (Brown and Holden, 1998; Thon *et al.*, 2000). Although several conditions were tested in this study, plasmid rescue and thus, gene sequencing were not successful. Most of the retrieved plasmids from ampicillin resistant colonies had a molecular weight similar to the transforming plasmid. It is possible that rearrangements of the plasmid during transformation or tandem integration of the plasmid sequences, as observed in the Southern blot, made rescue of the plasmid with flanking genomic sequences difficult. Tandem integrations are very common in transformation of filamentous fungi. Tandem integrations are suggested to be a result of circular plasmids undergoing homologous integration with each other to form circular oligomers, which could then integrate by homology with the chromosomal single copy, or a single copy of the plasmid may integrate, but the tandem repeats may then arise through secondary integration of further plasmid copies by homology with the first one (Fincham, 1989).

The possibility that suitably sized fragments were not retrieved during plasmid rescue could be due to a number of factors that include: 1) the large sizes of the restriction fragments generated by *Hpa*I, *Nhe*I, *Nru*I, or *Xba*I could have a more reduced ligation or transformation efficiency than smaller size fragments and therefore, could explain why only sizes corresponding to the molecular weight of the transforming plasmid were retrieved. 2) The antibiotic ampicillin resistance gene or plasmid replication origin of the transforming plasmid adjacent to the flanking sequences of the genomic DNA was disrupted during its integration into the *T. basicola* genome, whereas the sequences of copies of the transforming plasmid that integrated by homology probably remained intact and were possibly rescued.

Despite the unsuccessful attempts to rescue the plasmid with flanking genomic sequences, several phenotypes of the mutants, which could be involved in pathogenicity and fungal survival, have been determined.

The function of melanin has been previously studied using mutants and inhibitors of melanin production. The role of melanin has been reported to be an essential factor for host invasion in some phytopathogenic fungi by generating the appressorial turgor pressure required for successful penetration of leaves via infection pegs that protrude from the appressorial adhesive surface (Howard and Valent, 1996; Money, 1997). However, it is postulated that the role of melanin in pathogenicity in root invading fungi may not be for host penetration but for the production of dark runner hyphae on the root surface that some fungi use to spread along the root during infection (Henson *et al.*, 1999; Dufresne and Osbourn, 2001; Sesma and Osbourn, 2004) In this study, endoconidia were used as an inoculum for pathogenicity and, as reported in the literature, endoconidia are hyaline in culture (Nag Raj and Kendrick, 1975). The structures that develop from the germ tube tip of *T. basicola* endoconidia during the infection process have previously been reported to involve the formation of a slender penetration peg, which develops into an infection vesicle and differentiates into infection hyphae (Mims *et al.*, 2000). Whether all these structures contain melanin is not known, however, melanized chlamydospores are eventually produced at the reproduction stage. The infection hyphae of *T. basicola* could possibly act like the runner hyphae that use the host's nutrients during the biotrophic phase to synthesize melanin required for the production of chlamydospores rather than for host penetration. The heavily melanized chlamydospores are required for the survival of the fungus during adverse conditions (Tsao and Bricker, 1966). In this study, 24 h post-inoculation of cotton seedlings by wild-type strain and the reduced melanin mutants (P16, P849 and P954), were able to penetrate the root hairs and the epidermis of the tap root causing light brown lesions on the root surface. The reduced melanin *T. basicola* mutants

do not seem to be altered at this critical step of the infection process. Therefore, it is possible that the role of melanin in *T. basicola* is not required for host penetration but may be for later stages of the infection.

Non-melanized *G. graminis* mutants have been reported to be less resistant to hydrolytic host enzymes (Frederick *et al.*, 1999). The presence of melanin has been suggested to cause sequestration of enzymes secreted by the fungal pathogen. This property has been proposed to be a mechanism for localizing the pathogenic factors to the site of the infection (Jacobson, 2000). Punja (2004) also reported that *T. basicola* isolates with reduced pigmentation were weakly virulent on bean leaves compared to darkly pigmented *T. basicola* isolates. It is possible that the reduction of melanin in P16, P849 and P954 could hinder their resistance to hydrolytic enzymes that are secreted by the host. Further experiments would be required to determine the effect of hydrolytic enzymes, such as chitinase, on *T. basicola* reduced-melanin mutants compared to the wild-type.

In contrast to P16, P849 and P954, the mutants P737 and P888, appeared to be darker than the wild-type on PDA, suggesting that the production of melanin is enhanced for these mutants compared to the wild-type. The enhanced melanin mutants (P737 and P888) also behaved like the wild-type strain and the reduced melanin mutants, 24 h post-inoculation of cotton seedlings. A week after inoculation with the five mutants, spreading of the lesions was minimal with very few chlamydospores, whereas the wild-type strain had extensively invaded the root hairs and the tap root and produced numerous chlamydospores. It is possible that these mutants, like the reduced melanin *T. basicola* mutants are able to adhere to the surface of the root and penetrate. Once inside the host cells, like the reduced melanin *T. basicola* mutants, they may have reduced ability to continue the biotrophic stage and induce necrosis and produce further chlamydospores. This observation has also been reported by Dufresne *et al.* (2000) on a non pathogenic

Colletotrichum lindemuthianum mutant (H433) generated via insertional mutagenesis. The mutant strain, H433 germinated and produced normal looking appressoria that penetrated and infected epidermal cells causing brown lesions. Using cytological studies, Dufresne *et al.* (2000) were able to show that infection vesicles and primary hyphae developed and grew normally in infected epidermal cells. However, after a long incubation period, secondary hyphae were not visible in H433 whereas wild-type strain had invaded the infected tissue with secondary hyphae. Their cytological studies concluded that the development of the infection process by H433, on common bean was stopped at the switch between biotrophic and the necrotrophic phases and that a regulatory gene, *CLTA1*, was found to be responsible for blocking this transition.

T. basicola is also classified as a hemibiotrophic fungus because of the biotrophic phase followed by the necrotrophic phase. It is possible that the transition between biotrophy and necrotrophy is delayed in the *T. basicola* mutants during the infection process. During the biotrophic phase of the *T. basicola* mutants, it is possible that the infection intracellular hyphae are not extensively produced and therefore this reduced the rate of spread of lesions and the colonization of the roots. To confirm this observation for *T. basicola* mutants, cytological studies would be required to compare the development and the number of the infection hyphae of the wild-type strain to that of the mutants during infection time courses of hypocotyls segments of the cotton root. A number of differentially expressed genes have also been implicated to be essential for the successful colonization of hosts by fungi. These genes include isocitrate lyase, a component of the glyoxylate cycle that is involved in the metabolism of acetate and fatty acids (Idnurm and Howlett, 2002), *NUT1*, a major nitrogen regulatory gene (Froeliger and Carpenter, 1996) and *FOW1*, a gene that encodes a mitochondrial carrier protein required specifically for root colonization (Jacobson, 2000). Similar genes to those mentioned above and others could be possible pathogenicity factors yet to be identified in *T. basicola* mutants.

Increased levels of melanin have been reported for *G. graminis* mutants, which were found to be less pathogenic than the wild-type on rice. *G. graminis* heavily-melanized mutants were limited in their ability to secrete their own extracellular lytic enzymes required for host tissue degradation (Frederick *et al.*, 1999). Furthermore, enzymes such as cellulase and polygalacturonase have been reported to be inhibited by phenols found in melanins (Sineiro *et al.*, 1997). It is possible, therefore, that the amount of lytic enzyme secreted was similar in P737, P888 and the wild-type, but that enzymes were bound and inactivated by cell wall melanin after they were secreted by P737 and P888. The conduct of extracellular enzyme assays would be required to detect the enzymatic activity of these mutants compared to the wild-type. Genes that encode hydrolytic enzymes, such as endo- and exopolygalacturonases, pectate lyase and cellobiohydrases have been implicated during the advanced stages of the infection process, the necrotrophic phase and sporulation (Kahmann and Basse, 2001).

Melanin has also been implicated in providing cell wall rigidity that serves to limit permeability and facilitate osmolyte accumulation (Henson *et al.*, 1999). Osmolyte accumulation in the cell creates a differential in osmotic pressure that drives a net osmotic flux of water into the cell, preventing water loss and generating turgor pressure. Continuous water influx is necessary for hyphal growth and hyphal turgor is required for invasive growth through soils and the solid tissues of living plants or animals (Money *et al.*, 1998). The most apparent stimulus reported affecting cellular turgor is hyperosmotic stress (Talbot, 1995). The exposure of *T. basicola* mutants and wild-type to increased concentrations of sodium chloride reduced their ability to grow on PDA. There were, however, differences in the ability of the strains to respond to osmotic stress. *T. basicola* reduced melanin mutants (P849 and P954) grew faster than mutants with enhanced melanin (P737 and P888) but all grew slower than the wild-type at 2% sodium chloride. Only the growth rate of P16 was similar to the wild-type. The implication here in the

differences can be related to other effects caused by the mutation that can have profound effects upon cellular hydration or growth, more important than melanin content. This is because the production of melanin is not associated with the growth of the fungus and its synthesis is considered as secondary metabolism (Henson *et al.*, 1999).

To date, no research has been conducted to determine the level of melanin in *T. basicola*. This study should stimulate the need to quantify the level of melanin during growth of the mutants or wild-type in either PDB or PDA in order to confirm the observation of the reduced or increased melanin production for these mutants.

P16 also showed a significant reduction in the growth rate on PDA and water agar and produced the lowest number of endoconidia and chlamydospores when grown on PDA. The reduced growth might be associated with reduced mobilization of storage compounds for active growth. P16 also produced abnormal chlamydospores compared to the wild-type. Taken together, these results show that the mutation in P16 resulted in pleiotropic phenotypes affecting melanin biosynthesis, chlamydospore morphology, growth rate and conidiogenesis.

In conclusion, PEG-mediated transformation facilitated the production of five mutants with reduced pathogenicity. Although, the percentage of pathogenicity mutants obtained in this study is fairly high, these were not completely impaired in their pathogenicity. Therefore, differences in screening criteria between studies could result in differences in the percentage and type of mutants obtained. The *T. basicola* mutants displayed an array of phenotypes. Each mutant demonstrated a different overall phenotype and therefore, these mutations are likely to be a result of disruption of five different genes. Southern hybridisation analysis confirmed the integration of the transforming plasmid DNA in all the five pathogenicity mutants. These integrations possibly occurred at different locations

in the genome. Four of the mutants showed single sites of insertion and one mutant had multiple site insertions, but they all had one or several copies of DNA in some of the sites.

The *T. basicola* pathogenicity mutants appear to germinate, grow towards the host and penetrate but cause reduced lesions. They appear to be unable to sustain a durable compatible interaction within the host environment and therefore unable to effectively colonise their host. Some of the mutants showed reduced melanin whilst others showed enhanced melanin. The production of melanin in this fungus may not play a role for penetration but may be required for the later stages of infection. Some of the mutants demonstrated better adaptation to osmotic stress than others. It is also possible that these mutants are unable to secrete sufficient enzymes to fully detoxify the plants chemical defences once inside the host tissue. Therefore, all these factors suggest that the mutations probably affected genes that are triggered during the later stages of the infection. Genes also affected probably include structural genes for transport and nutrient metabolism that resulted in reduced pathogenicity. It is also possible that signaling genes, similar to heterotrimeric G proteins, mitogen-activated protein (MAP) kinase and/or cyclic AMP-dependent protein kinase genes are affected. The disruption of these genes has been reported to regulate a number of key steps during plant infection by root invading fungi. These include; osmotic stress, invasive growth and sporulation, expression of cell wall degrading enzymes and pathogenicity (Tudzynski and Sharon, 2003). The different effects on relative pathogenicity on cotton and lupin in one of the *T. basicola* pathogenicity mutants may also allow identification of host-specific factors.

Finally, this study has detected some potential problems in using PEG-mediated transformation as a general mutagenesis/gene tagging procedure in *T. basicola*, due to possible tandem integrations. Therefore, to investigate whether or not the altered phenotypes of these mutants are linked to the plasmid insertion site can be time

consuming. However, the mutants produced in this study provide a powerful tool in future studies of this fungus to further understand the critical key factors involved in its pathogenicity

Chapter 5 . General Discussion

This study was part of a multidisciplinary research approach aimed to identify pathogenicity factors required for the infection of *T. basicola* on cotton in order to reduce the impact of black root rot on the cotton industry in Australia. The first aim of this project was to develop bioassays to determine whether the aggressiveness of *T. basicola* towards plants is host-specific and whether a correlation exists between its ability to germinate and grow towards its hosts and its ability to cause disease. The other aim was to generate mutants of *T. basicola* with reduced pathogenicity towards cotton by developing a genetic transformation protocol. The ability of *T. basicola* to recognise and penetrate a specific host, exploit its nutrients, overcome its innate defense responses and cause disease relies on a number of signalling mechanisms. A genetic transformation approach has been proven by several studies to be the most effective strategy to study the disease causing mechanisms of plant pathogenic fungi and has led to the identification of genes related to pathogenicity and their regulation of stages in the infection and host colonisation processes. Identification of genes operating during important stages of cotton infection by *T. basicola* will enable the control of black root rot by developing genetically modified cotton with increased resistance to the disease.

5.1. *T. basicola*-host specificity

5.1.1. Pathogenicity

T. basicola has been reported to display host recognition and specificity at the species and cultivar level. However, whether this specificity involves host-specific stimuli that trigger the germination of spores and hyphal growth towards the host has not been investigated. This study first investigated the pathogenicty of *T. basicola* strains towards a variety of plants. Although in this study the *T. basicola* isolates did not show differences in their

virulence to lupin and pansy seedlings used at an inoculum concentration of 3.5×10^5 endconidia/mL, they behaved differently in their ability to cause lesions on other plants (Chapter 2). These variations could be attributed to genetic differences between the *T. basicola* strains causing them to behave differently towards different plants. These observations lend support to the concept that a "universal pathogenicity" gene probably does not exist and that a wide variety of genes determine pathogenicity and that the importance of these genes in exerting aggressiveness differs between host species.

Evidence of such host specificity was also observed in the *T. basicola* mutant, P737. The *T. basicola* cotton isolate (BRIP40192) was highly pathogenic to cotton roots as well as lupin roots (Chapter 2). P954, a mutant of BRIP40192 also showed reduced pathogenicity towards cotton roots (Chapter 4) but as the wild-type, it was relatively virulent towards lupin seedlings (Chapter 4). However, P737 behaved differently from the wild-type towards cotton and lupin (Chapter 4) and may be affected in host-specificity related genes.

Fungal-host specificity is an interaction that is exemplified by the "gene for gene" interaction between the products of dominant plant resistance genes and corresponding avirulence genes of the pathogen. It is possible that host-specific signals are related to the ability of *T. basicola* to cause disease on some hosts and not on others. Although the molecular mechanisms that determine host specificity in many filamentous fungi are not fully understood, a number of virulence determinants are reported to be host specific. For example, host-specific toxins were among the first pathogenicity determinants confirmed by molecular genetics (Tudzynski and Sharon, 2003).

5.1.2. Endoconidial germination and directional hyphal growth

The events involved in the successful invasion of a plant are not only related to penetration and the ability of the fungus to cause disease but also related to germination and growth of the fungus towards the plant.

This study sought to investigate using seedlings of various plants, whether *T. basicola* hyphal germination and growth was specifically directed towards host roots or whether their growth was random. The chemical signals such as nutrients (sugars and amino acids) exuded from roots are only thought to serve as general germination stimuli that have the potential to cause orientation of a hypha towards nutrients. This has been studied *in vitro* in arbuscular mycorrhizal fungi which include *Gigaspora gigantea* (Suriyapperuma and Koske, 1995) and *Glomus mosseae* (Vierheilig *et al.*, 1998). However, the nature of the stimulatory signals and the host specificity of the signals are unknown in most fungi studied. The elucidation of specific stimulatory signals would clarify the events and specificity involved in fungal recognition of potential hosts at this stage of fungal-host interaction.

Since evidence of host specificity was apparent between different *T. basicola* strains in their ability to inflict damage on different hosts, it was hypothesized that different strains of *T. basicola* hyphae would not exhibit random growth, but rather *T. basicola* endoconidia would grow towards roots in response to specific-host stimuli. It was also hypothesized that different strains would respond differently to different hosts. This study provided ample evidence that the growth of *T. basicola* hyphae towards host root exudates was not a random occurrence but that the germination and growth of hyphae was directed towards all seedlings tested (Chapter 2). However, this directionality was probably not due to apparent host-specific stimuli. For example, the cotton isolate did not only grow towards its respective host, cotton seedlings, but also demonstrated strong directional growth towards

lupin (susceptible host plants), and lettuce (non-susceptible host plant) and also towards broccoli and wheat seedlings (non-susceptible host plants) (Chapter 2). Such a phenomenon has also been previously reported whereby root exudates of both host or non-host plants can non-specifically trigger germination and hyphal growth of spores of fungal root pathogens (Lockwood, 1977; Deacon, 2006). Host preference tests conducted in this study suggest that the quantity of root exudates may play a role in triggering germination and hyphal directional growth. For example, *T. basicola* isolates demonstrated faster directional growth towards non-susceptible hosts than their respective hosts, such as the lettuce1 isolate on broccoli and wheat seedlings (non-susceptible host plants) (Chapter 2). Future approaches on the quantification and identification of exudates released from seeds will greatly help to identify the compounds crucial for the stimulation of *T basicola* germination and directional attraction and thus factors related to host specificity.

However, these experiments cannot effectively conclude that host-specific stimuli such as plant-derived compounds beyond sugars or amino acids are not involved in the stimulation of spore germination and directional attraction by plant root exudates. For example, Ruan *et al.* (1995) observed that the addition of a plant-derived flavonoid in minimal medium caused the fungus *F. solani* to germinate at a much higher frequency. They concluded that flavonoids, including defence-related isoflavonoid phytoalexins identified in root exudates of legumes, are a notable example of a host-specific signal because they have the ability to induce spore germination and pathogenicity of *F. solani*. The stimulatory effect of germination by flavonoids was found to be through a protein-kinase A (PKA) dependent pathway, in contrast to the PKA-independent pathway of nutrients. Such specific signalling has the inherent specificity that is necessary to explain the selective nature of fungal diseases of plants. It is possible that specific compounds isolated and identified such as flavonoids in exudates of legumes (host plants of *T. basicola*) other than nutrients (sugars and amino acids) could be tested to stimulate germination of *T. basicola* endoconidia in the

future. However the conduct of such assays would not be possible for the determination of specific signals from host plants such as cotton. There is limited information published on specific compounds that have been isolated and identified from cotton root exudates. If such compounds from cotton exudates specific to *T. basicola* are identified, then inhibitors may be designed to bind to their products and block the growth of *T. basicola* without affecting the plant.

5.1.3. Pathogenicity and directional growth

The growth of hyphae of different *T. basicola* strains towards host plants provided information as to whether it is related to the ability to cause lesions. For example, *T. basicola* isolates that exhibited a strong directional growth towards plants did not necessarily cause disease and isolates that severely infected susceptible plants demonstrated a weak directional growth response. This suggested that screening for *T. basicola* pathogenicity mutants is likely to be more informative than screening for directional growth mutants. This is because directional growth seems to be weakly related to the ability to cause disease on a particular host and therefore, genes with practical outcomes are more likely to be found if the primary screening is for actual virulence.

5.2. Development of a PEG-mediated transformation protocol for *T. basicola*

There were no previous reports of successful genetic transformation of filamentous fungi that were closely related to *T. basicola*, at least at the level of Order. The only available molecular tools used in *T. basicola* were based on PCR technology for phylogenetic studies (Paulin-Mahady *et al.*, 2002). Furthermore, no protocols for the isolation of *T. basicola* protoplasts had been developed. Due to these limitations, a basic and most common protocol, PEG/ $CaCl_2$ method successfully used previously for the transformation of many filamentous fungi was developed to produce *T. basicola* transformants for the first

time. Given the constraint of time, there was not sufficient data to effectively conclude that PEG-mediated transformation of *T. basicola* has an advantage of obtaining single gene integrations and that these integrations occurred at different chromosomal locations. However, preliminary data suggested that was the case. The major disadvantage of this technique was that the integration of the plasmid in the fungal genome may have occurred in tandem arrays which made plasmid rescue difficult and time consuming. Although the frequency of producing stable transformants using the protocol developed here was relatively low compared to other filamentous fungi using PEG-mediated transformation, a high proportion of transformants were found to be pathogenicity mutants using screening assays.

The *T. basicola* pathogenicity mutants possibly showed single insertions, however, it is difficult to conclude that the insertions occurred in different chromosomal locations due to possible tandem integrations. The random disruption of genes resulted in reduced pathogenicity, as well as pleiotropic effects on cellular processes, including conidiation, growth rate and melanin synthesis. Some of these genes could very well be regulatory genes that play important roles in pathogenicity. Genes involved in the differences of pathogenicity of the wild-type and P737 on cotton and lupin could be important for finding genetic determinants of host specificity, for example, host selective toxins.

Because of technical limitations, such as low transformation rates of stable transformants and plasmid rescue difficulty, other approaches of increasing the numbers of stable transformants and rescuing the flanking sequences adjacent to the plasmid should be further investigated. Strategies to increase the transformation frequency of stable transformants could include increasing the amount of the transforming plasmid DNA. However, one could argue that the transformation frequency in *T. basicola* may not be constrained by DNA uptake, as a very high number of transformants were obtained on

selective medium, but rather by the efficiency with which the plasmid DNA is integrated into the genome that caused more than 99% of these transformants to abort their growth (Chapter 3). Monfort *et al.* (2003) reported that the transformation of *Mucor miehei* resulted in plasmid deletion and phenotypic instability and that this could be attributed to the coenocytic nature of its mycelium and the action of defence mechanisms that present the establishment of foreign DNA. It is possible that the loss of the plasmid DNA in *T. basicola* abortive transformants is due to the biological nature of *T. basicola* and its ability to detect and eliminate foreign DNA. Factors that could increase the transformation frequency of stable transformants include the incorporation of DNase inhibitors or increasing the number of protoplasts. Increasing the number of protoplasts is strongly recommended as it was observed that higher numbers of regenerated protoplasts gave a higher transformation frequency of stable transformants (Chapter 3). However, maintaining the appropriate ratio of protoplasts to plasmid DNA is relevant to the success of transformation (Mishra, 1985).

An inverse PCR technique could be used as an alternative strategy to rescue the sequences flanking the plasmid DNA (Keim *et al.*, 2004). However, this technique can also be time consuming. This is because it is not known which sequences of the transforming plasmid DNA are adjacent to the genomic DNA. Therefore, several forward and reverse primers would have to be designed around the plasmid DNA.

5.3. Screening of *T. basicola* transformants

Although large numbers of transformants, such as 10^5, are needed to saturate the genome with single insertions at different loci (Kang and Metzenberg, 1993), in this study only 202 transformants were required to generate five pathogenicity mutants (Chapter 4). The fact that none of the 202 *T. basicola* transformants were affected in their ability to grow towards cotton roots, probably implies that fewer genes are involved in hyphal directional

growth than genes that contribute to disease unless mutations that affect directional growth are lethal. Another possibility could be that more than one gene needs to be disrupted or knocked out to produce an altered directional growth phenotype. This is because it may prove difficult to demonstrate any links between defects in directional growth and pathogenicity if mutants with individual enzyme defects were isolated. Such difficulties and discrepancies have also been indicated in studies whereby mutants that were defective in single enzymes failed to show a correlation between their activities and their involvement in pathogenicity (Annis and Goodwin, 1997).

5.4. Alternatives to PEG-mediated transformation

Alternatives to increase transformation frequencies and to facilitate the rescuing of the plasmid DNA, include the development of other random insertional mutagenesis techniques, such as REMI and ATMT. In REMI, (a protoplast-PEG-based protocol), the fungus is transformed with plasmid DNA in the presence of a restriction endonuclease that generates compatible cohesive ends between the chromosome and plasmid DNA, resulting in plasmid integration at the corresponding restriction sites in the genome (Kahmann and Basse, 1999). Although REMI favours monolocal integration, the major disadvantage is a non-random distribution of integration sites and an unexpectedly high percentage of unsolicited genomic deletions and rearrangements resulting in the generation of untagged mutations (Kahmann and Basse, 1999) that call into question the usefulness of REMI for random insertional mutagenesis. It has been proposed that cutting of the chromosome by a restriction enzyme followed by imperfect DNA repair during transformation is responsible for untagged mutations (Sweigard *et al.*, 1998).

Agrobacterium tumefaciens, a widespread tool used to transfer DNA to plants has also been used to transform both yeast (Bundock *et al.*, 1995) and filamentous fungi (de Groot *et al.*, 1998), among them several phytopathogenic ascomycetes (Mullins *et al.*, 2001; Rho

et al., 2001). ATMT has been shown to be more promising and reliable offering profound advantages over all other genetic transformation protocols. It can transform hyphae, spores and blocks of mushroom mycelial tissue, therefore, eliminating the need to generate protoplasts (Mullins and Kang, 2001). In addition, it produces a high frequency of single tagged insertion events and transformation efficiencies can be up to 600 times higher than PEG-mediated transformation and REMI (Zwiers and De Waard, 2001; Leclerque *et al.*, 2004).

5.5. Alternatives to random insertional mutagenesis

Alternatives to random insertional mutagenesis include expression pattern approaches for gene identification and proteomics.

5.5.1. Expression pattern approaches

These approaches include differential/subtractive cDNA (complementary DNA) hybridisation techniques, differential display of mRNA (messenger RNA), cDNA Amplified Fragment Length Polymorphism Analysis (cDNA-AFLP), Expressed Sequence Tag sequencing (ESTs) and microarrays. These various strategies allow the identification of fungal genes that are preferentially expressed during pathogenesis (Gold *et al.*, 2001; Massart and Jijakli, 2006). However, these approaches cannot assess the requirement for pathogenicity of the gene. Therefore, the isolated gene is cloned and targeted gene disruption is used to generate a mutant in order to evaluate its role in the phenotype of interest. Gene expression pattern approaches are only useful for the application of host and pathogen where their genomic sequence data is available but in the case of *T. basicola*, such techniques are not possible as *a priori* knowledge of *T. basicola* genome sequences is still lacking.

5.5.2. Proteomics

Proteomics is another alternative to random insertional mutagenesis. Although this technology does not require *a priori* knowledge of the genome sequence of *T. basicola*, the protein identified would not be in its complete sequence form and therefore genes involved may be difficult to identify. Furthermore, this technique would not provide sufficient information on the function of the protein unless targeted gene knockout studies are performed.

5.6. Possible genes related to pathogenicity in *T. basicola* mutants

The proportion of pathogenicity mutants (2.5%) obtained in this study is similar to the findings of Idnurm and Howlett (2001). In their review article of fungal pathogenicity genes, they concluded that the number of fungal pathogenicity mutants obtained through random insertional mutagenesis is approximately 0.5-2%, implying that a phytopathogenic fungus may have between 60 and 360 pathogenicity genes. Therefore, it is possible to suggest that *T. basicola* could have a similar or higher number of pathogenicity genes than 360.

T. basicola pathogenicity mutants were affected in melanin production (lower or higher than the wild-type), their ability to expand their lesions and to tolerate osmotic stress. Some also showed reduced conidiation, reduced growth or abnormal morphology (Chapter 4).

Idnurm and Howlett (2001) have reviewed fungal pathogenicity genes and categorised them into various groups according to the different stages of the infection process. These groups are briefly discussed below in order to postulate possible putative gene homologues

of other fungal pathogens in relation to *T. basicola* pathogenicity mutants produced in this study.

5.6.1. Production of infection structures

The production of infection structures is essential for the successful penetration into host plants by several phytopathogenic fungi including *M. grisea*. These fungi require melanin in their infection structures (appressoria) to enable the penetration peg to puncture the plant epidermis. Melanin-deficient mutants of *M. grisea* are unable to penetrate the plant epidermis and are therefore non-pathogenic (Chumley and Valent, 1990). On the other hand, the presence of melanin in root invading fungi may not be required for host penetration. *T. basicola* is not known to produce appressoria or other specialized infection structures (Hood and Shew, 1997b). Therefore, the genes disrupted in the *T. basicola* pathogenicity mutants are unlikely to belong to this group.

5.6.2. Cuticle and cell wall degradation

A number of enzymes such as cutinases, pectinases or endopolygalacturonases that are necessary for the degradation of plant cuticle and cell walls and other physical barriers have been implicated in the pathogenicity of fungi. It is possible that in the *T. basicola* pathogenicity mutants, the loss or disruption of genes involved in the production of these enzymes are affected. This would slow down the process of degradation and therefore reduce the rate of colonization and in turn reduce the size of lesions compared to the wild-type. However, the primary mutation is unlikely to be in one of the genes in this group as other defects in melanin synthesis (lower or higher than the wild-type), conidiation, growth rate, chlamydospore morphology or osmotic pressure were apparent.

5.6.3. Responding to host environment

Once inside the host environment, fungi produce enzymes that detoxify plant toxins (such as phytoanticipins and phytoalexins) in order to invade and colonise the plant (Osbourn, 1996). The genes that encode these enzymes may have been disrupted in the *T. basicola* pathogenicity mutants. Such a mutational effect could also slow down the function of the enzymes involved in the degradation of the toxins secreted by the host. This also could explain why the size of the lesions is smaller than the wild-type.

In addition to the production of enzymes by fungi that detoxify plant toxins, a fungus can encounter other challenges during growth in *planta*. For example, some non-pathogenic mutants have been shown to have defects in genes that are involved in primary metabolism. Such genes have also been implicated in pathogenicity and have been induced during *in vitro* starvation conditions (Kahmann and Basse, 2001). For example, the regulation of certain compounds such as carbon or nitrogen assimilation is important for disease development by phytopathogenic fungi on leaves and roots (Idnurm and Howlett, 2001; Kahmann and Basse, 2001). It is possible that the *T. basicola* pathogenicity mutants could not effectively metabolise certain nutrients from the host and could explain their reduced ability to sustain a durable compatible relationship within their host.

However, it is unlikely that a direct mutation of genes involved in the biosynthesis of fungal toxins or genes involved in primary metabolism will also result in pleiotropic effects (melanin synthesis, conidiation, growth rate, spore morphology or osmotic pressure) exerted by the *T. basicola* pathogenicity mutants.

5.6.4. Fungal toxins

Examples of fungal toxins that have been implicated in the pathogenicity of fungi include host-specific toxins such as HC toxins in *C. carbonum* (Ahn *et al.*, 2002). To date, there

have been no reports of toxins generated by *T. basicola*. Therefore, it is speculative to suggest whether the mutation in the *T. basicola* pathogenicity mutants occurred in genes involved in the biosynthesis of toxins. However, this assumption should not be ignored, but instead the production of toxins by *T. basicola* should be investigated.

5.6.5. Signalling genes

Signalling genes are the largest group of pathogenicity genes. They include regulatory genes that encode the MAPK, cAMP-PK and the heterotrimeric G proteins involved in signalling cascades. The disruption of these signaling genes has been shown to regulate diverse processes in filamentous fungi such as growth, osmotic stress response, infection structure formation, invasive growth, toxin production, expression of surface hydrophobins, conidiation, and pathogenicity (Idnurm and Howlett, 2001). Therefore, there is a very high likelihood that the mutational effects in the *T. basicola* pathogenicity mutants are a result of the disruption of signaling genes. The disruption of putative signaling gene homologue in *T. basicola* pathogenicity mutants could have produced plieotropic effects such as reduced growth rate, reduced conidiation, low osmotic stress response, reduced invasive growth, decreased toxin synthesis, as well as reduced pathogenicity.

5.6.6. Novel and unclassified pathogenicity genes

A number of novel pathogenicity genes have also been identified and these include genes that either have no homologues in gene databases or reveal new mechanisms on the plant-pathogen interaction (Idnurm and Howlett, 2001). It may be possible that the genes disrupted in the *T. basicola* pathogenicity mutants are novel genes that are affected in the formation of cell wall structure which decreased or increased the accumulation of melanin synthesis. This led to the production of *T. basicola* pathogenicity mutants with reduced or enhanced melanin mutants. However, melanin may not be required for penetration as both

reduced and increased melanin *T. basicola* mutants, demonstrated similar lesions to the wild-type during the initial stages of the infection. The presence of melanin may be required for later stages of the infection to provide protection against host defences or to confer cell wall rigidity for invasive growth and survival to effectively cause pathogenicity on its hosts. This could explain why the reduced melanin *T. basicola* mutants were unable to effectively colonise the roots and thus led to a reduction in pathogenicity. So why did the enhanced melanin mutants also show reduced pathogenicity? Constitutively melanised mutants of *G. graminis* have been shown to produce less extracellular cellulose, pectinase, protease and laccase. It has been proposed that constitutive melanisation limits secretion of lytic enzymes necessary for host tissue degradation and subsequent pathogenesis (Henson *et al.*, 1999).

5.7. Future directions

Previous studies on the ability of *T. basicola* to cause black root rot started with cytological and microscopical observations. This study attempted to pursue the understanding of *T. basicola* pathogenicity at a further level by trying to gain a foothold on the genes related to its pathogenicity. *T. basicola*-host specificity studies were conducted and a transformation protocol for the isolation of *T. basicola* pathogenicity mutants was achieved. Further opportunities to improve and progress our understanding of the factors involved in controlling black root rot that are worthwhile investigating in the future are described below. However, these will not lead to the conclusive identification of functions involved in *T. basicola* pathogenicity unless the disrupted genes of the *T. basicola* pathogenicity mutants generated in this study are identified and more molecular biology tools are developed for this pathogen. Moreover, unravelling the genome sequence of *T. basicola* and cotton will significantly help define the genetic information required for pathogenicity and host-specificity.

5.7.1. Biological control

The selection of non-susceptible host plants such as broccoli and wheat could be exploited in disease management (traditional crop rotation) to induce the germination of *T. basicola* spores but prevent the fungus from surviving. This is also known in part as biological control. Understanding how non-susceptible host plants exert their protective effects can be used as a prerequisite to understand plant-fungal interactions.

5.7.2. Cytological and microscopy studies

Previous cytological and microscopical studies of the infection process by *T. basicola* were limited to susceptible hosts (Hood and Shew, 1997b; Mims *et al.*, 2000). Future work could also involve the same procedure of Hood and Shew (1997b) that utilises non-destructive light microscopy to document the timing and sequence of events that occur from the moment the *T. basicola* isolate contacts a susceptible host root hair until the time an infection vesicle develops within the same root hair. In addition, the formation of the infection structures and the development of the infection process can be compared to non-susceptible hosts or non-host plants. Cytological and transmission electron microscopy studies employed by Mims *et al.* (2000) can also be used to study the initial penetration and examine the development of the infection structures inside the cell lumen of not only the host, but also a non-susceptible host or a non-host plant during the infection. This will provide exquisite knowledge at which stage the infection process is blocked in wild-type *T. basicola* strains associated with non-susceptible hosts or non-host plants.

Light microscopy, cytological and transmission electron microscopy studies mentioned earlier can also be applied to *T. basicola* (cotton isolate) and compared with *T. basicola* pathogenicity mutants. This will determine at which stage the infection process is delayed after penetration into the plant root.

5.7.3. Proteomics

Proteomic analyses are complementary to genomic approaches. To date, most of the proteomic studies in plant pathogenic fungi have been conducted using 2-D (dimensional) gel analysis (Xu *et al.*, 2006). It is anticipated therefore that 2-D protein gel analysis can be used to identify differentially expressed proteins expressed during pathogenicity in order to understand why some *T. basicola* isolates are aggressive towards some hosts and not towards other hosts. Furthermore, protein expression profiles can also be used to determine expression of *T. basicola* proteins in susceptible hosts and non-susceptible hosts and non-host plants.

5.7.4. GFP technology

The development of a PEG-mediated protocol transformation for *T. basicola* with the transforming plasmid DNA, pGpdGFP, containing a gene encoding GFP (green fluorescent protein) offers the advantage that the GFP can be used as a fluorescence cytological marker for studying disease progression of *T. basicola* mutants not altered in pathogenicity and compared to pathogenicity mutants in cotton plants. This technology has been applied in *Leptosphaeria maculans* using the same plasmid pGpdGFP (Sexton and Howlett, 2001). However, the hyphae of all 22 selected transformants used by Sexton and Howlett (2001) had low levels of fluorescence. In this study, a preliminary attempt to visualise six stable transformants not altered in pathogenicity grown as germinating endoconidia for 3 h in 3 mL of PDB and observed under an Olympus BH-2 microscope (using a blue BP-490 exciter filter and dichroic mirror) was unsuccessful. Large numbers of transformants would be required to be viewed under the microscope as regulation of the constitutive promoter, glyceraldehyde-3-phosphate dehydrogenase (P*gpdA*), can vary between transformants. Such variation could be a result of differences in sites of integration into the genome or differences in copy number of the introduced *egfp* gene between transformants. It may also be possible that *PgpdA* cannot be expressed in *T. basicola* altogether. A highly

recommended plasmid for the visualisation of fungi in *planta* is pCT74 which expresses SGFP (contains a serine to threonine substitution at amino acid 65 as well as plant-optimised codon usage that also detects a cryptic intron splice site reported to reduce GFP expression in *Arabidopsis*) under the control of the *ToxA* gene promoter from *Pyrenophora tritici-repentis* (Lorang *et al.*, 2001). This promoter is reported to confer versatile, high level constitutive gene expressive as compared to other GFP expression vectors that harbour *Aspergillus*, *Neurospora* or *Colletotrichum* promoters. The *ToxA* gene promoter has been proven to be useful for expressing GFP in eight different genera of filamentous fungi all belonging to the Ascomycota (Lorang *et al.*, 2001).

Other advantages of GFP as a reporter gene include the use of nutrient-regulated promoter-GFP fusions to sense the fungal microenvironment in *planta* (Rohell *et al.*, 2001). This will enable an understanding of how fungal metabolism changes during host colonisation and its importance in establishing the role of nutritional factors that trigger the infection process or the formation of infection structures. Such an approach may lead to the development of new control strategies for black root rot.

5.7.5. *In vitro* bioassays

This study isolated *T. basicola* pathogenicity mutants that appeared to be affected in their melanin biosynthesis. Some mutants showed reduced and others showed enhanced melanin synthesis. This observation should encourage future work to quantify the melanin content present in the mutants and comparing it to the wild-type.

There are several different pathways for fungal melanin biosynthesis, but the best characterised and most commonly utilised pathway is the dihydroxynaphthalene (DHN)-melanin pathway (Freeman and Ward, 2004). Studies with melanin inhibitors have shown that the DHN-melanin pathway is used by many filamentous fungi including *G. graminis*

(Freeman and Ward, 2004). Therefore, it is possible that the use of such inhibitors could elucidate the pathway/s used by *T. basicola* for melanin biosynthesis.

The isolation of reduced melanin and enhanced melanin pathogenicity mutants offers several opportunities to further characterise the role of melanin required for *T. basicola* survival or pathogenicity on cotton. For example, exposure of these mutants to ultra violet radiation, temperature, lytic enzymes, oxidative stress, desiccation, autolysis or metal cations could determine whether reduced melanin mutants are more susceptible to these factors than enhanced melanin mutants. All these factors and others have been investigated in many melanised and non-melanised pathogenicity mutants of filamentous fungi including *G. graminis* (Frederick *et al.*, 1999) in order to explain reduced virulence. Testing for nitrogen, carbon or acetone and fatty acid metabolism defects or their enzymes should be preformed to further analyse and compare fungal growth (assessed by radial growth assays) and sporulation between the two sets of pathogenicity mutants and comparing them with the wild-type to determine their role in reduced virulence. Such assays have been conducted in *M. grisea* (Froeliger and Carpenter, 1996; Balhadere *et al.*, 1999) or *L. maculans* mutants (Idnurm and Howlett, 2002) in which some of the genes involved in the metabolism of nitrogen, carbon or acetone and fatty acids have been identified using insertional mutagenesis.

5.8. Conclusion

The first step in characterising genes potentially involved in pathogenicity is to isolate and identify them. Which strategy is most appropriate depends to some extent on previous results from other phytopathogenic fungi and the objectives of the study. The random insertional mutagenesis strategy would be advantageous if the aim is to acquire genetic knowledge of an organism in which the genomic sequence is not known as in the case of *T. basicola*. Therefore, the development and application of an efficient DNA transformation

protocol is a fundamental and powerful approach to reveal genes of potential importance. However, to date, this objective remains elusive and remains one of several limitations to be resolved for many other phytopathogenic fungi. Nevertheless, this study has established that *T. basicola* can be transformed by the PEG/ $CaCl_2$ method paving the way for the isolation of genes involved in pathogenicity. Although the key to mutational strategies is the recovery of the affected genes, the task of recovering such genes using plasmid rescue in *E. coli* and the usage of DNA complementation assays to verify the mutation is extremely challenging, but may conceivably be achieved in the near future.

Finally, the continued development of molecular tools and *in vitro* bioassays in combination with classical cytological analysis and microscopy for *T. basicola* pathogenicity mutants offers promising opportunities to further dissect and understand the pathogenicity of this economically important fungus.

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