Cotton Catchment Communities CRC

FINAL REPORT

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

Cotton CRC Project Number: 1.1.21

Project Title: Molecular factors determining Thielaviopsis basicola-cotton interactions leading to Black Root Rot disease

Project Commencement Date: 01/07/2004 **Project Completion Date:** 30/06/2007

CRC Program: The Farm

Part 2 – Contact Details

Administrator:	Ms Carol Handebo, Research Office	
Organisation:	University of New England	
Postal Address:	Research Services, University of New England, Armidale, NSW, 2351	
Ph: 6773 3715	**Fax:** 6773 3543	**E-mail:** chandebo@une.edu.au
Principal Researcher:	Dr. Lily Pereg-Gerk, Senior Lecturer	
Organisation:	University of New England	
Postal Address:	School of Science and Technology, UNE, Armidale, NSW 2351	
Ph: 6773 2708	**Fax:** 6773 3267	**E-mail:** lperegge@une.edu.au
Supervisor:	(Name & position of senior scientist overseeing the project).	
Organisation:		
Postal Address:		
Ph:	**Fax:**	**E-mail:**

Signature of Research Provider Representative: _____

Part 3 – Final Report Guide (due within 3 months on completion of project)

(The points below are to be used as a guideline when completing your final report.)

Background

1. Outline the background to the project.

Starting in 2003, our group at UNE (L. Pereg-Gerk in collaboration with M. Katz and D. Backhouse) has been focusing research efforts on *Thielaviopsis basicola*, the cause of black root rot, because of its agricultural importance. We started with securing a competitive VC-funded postdoctoral fellowship from UNE (for three years, 2003-2005, to employ J. Baker and afterwards J. Coumans-Moens), which included limited support for operating expenses from UNE and with involving postgraduate and Honours students in the project (self-funded). To enable a faster rate of progress in the research and the development of required methodologies for a larger-scale project, we approached the CRDC for additional funding to support operating expenses for a seed project. These additional resources allowed us to achieve our goals in developing both the genetic tools for work with the fungal pathogen and the proteomics tool for analysis of both pathogen and its host - cotton.

Black root rot is a seedling disease caused by the soil-borne fungal pathogen *Thielaviopsis basicola*. It has been considered a significant threat to cotton and other crops in Australia, especially in cooler areas and seasons. The pathogen, *T. basicola*, produces thick walled spores that can survive in the soil for years (Figure 1). Although it was first detected in NSW in the 1980s it quickly spread by movement of the spores attached to footwear or machinery wheels and is now widespread in all cotton growing areas of NSW. In just over a decade it has come to affect more than half of the cotton farms in southern Queensland and New South Wales. Regular disease surveys of cotton fields in NSW have shown an increase of incidence from 22% of fields inspected in NSW in 1995 to over 60% of farms surveyed in NSW in the 2000/2001 season (Allen and Lonergan 1997; Allen 2002; Nehl and Allen 2001) and the incidences of black root rot in 2004 have reached 95% of the fields regularly surveyed in northern NSW. A report by Rourke and Nehl (Australian Cotton CRC, March 2001), indicating that yield losses of 25 to 50 % have been recorded in severely affected crops, as well as the limitation in management options for black root rot, highlight the importance of research into this disease.

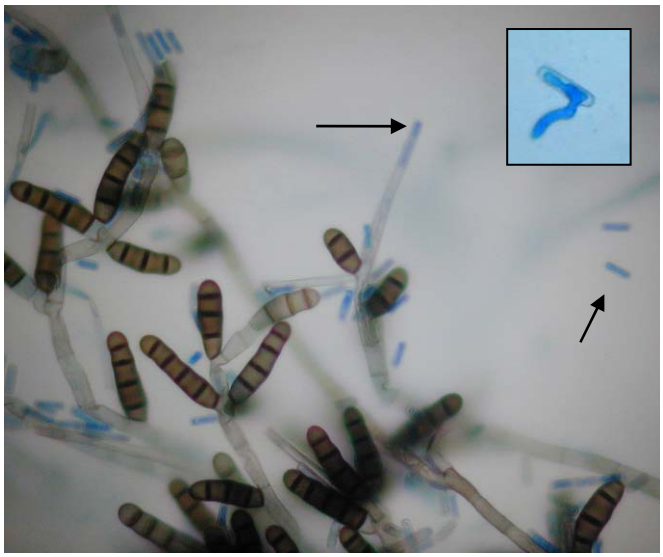

Figure 1. *T. Basicola* in a culture, showing two types of spores: conidiospores (shown with arrows) and dark, thick-walled, chlamydospores in chains. A germinating conidiospore is shown at the right top corner.

Chlamydospores are released into the soil and can survive dormant in the soil for years before infecting a host plant!

Photo taken by Dr Judy Baker, a former postdoctoral fellow in our group.

According to a comprehensive literature review (J. Baker and L. Pereg Gerk, unpublished) on Black Root Rot in plants such as cotton, pansy, red clover, cucumbers, carrots, strawberries and tobacco, (a) the infection mechanism has been well documented in tobacco and pansy using microscopy, (b) the effect of the disease on different plants is described, (c) there are some observations regarding the optimal conditions for an outbreak of the disease (d) some information exists on the biology of the pathogen as well as (e) some phylogenetic and taxonomic evaluations.

While improved field management and sanitation techniques to control black root rot as well as improvement in the tolerance of cotton varieties to diseases are currently under development, there is insufficient knowledge about (a) the control of spore germination, (b) the initial attraction of the different fungal strains to the plant roots, (c) their initial attachment to the plant roots, (d) the initial infection process and (e) the interactions of the microorganism with susceptible and resistant plants.

Although *T. basicola* has a broad host range, individual strains are only capable of infecting a limited number of host species. This indicates the widespread occurrence of resistance mechanisms in plants, including cotton, that are effective against many strains of the fungus and which are presumably based on recognition events at the molecular level. Resistance mechanisms to black root rot (or to wilts), especially at the level of the initial infection process, have not been investigated by modern molecular technology and progress in this field has been stalled for about 20 years. Now that tools are available, we proposed to employ a molecular approach to study the infection process. Proteomics and molecular genetics have been used in other systems to study microbial interactions with higher organisms, such as animals and plants. However, no molecular genetics or proteomics tool (with the exception of total DNA extraction from the fungus and PCR studies) was available for *T. basicola* at the commencement of this project, nor any protein map from cotton roots. This project was designed and has been undertaken as a seed project to develop tools towards the investigation of *T. basicola*-cotton interactions on the molecular (genetics and proteomics) level as well as in systems that allow large scale experiments.

Our long-term objectives have been to be able to better control black root rot by first, learning (1) what triggers germination and virulence in the plant pathogen, *T. basicola* (2) how does the plant (cotton) respond to the pathogen, and then finding out how to control the pathogen-cotton interactions in a way that will contribute to sustainable management of this disease.

In the seed project (1.1.21) we aimed at developing experimental procedures (material and methods in molecular genetics and proteomics) for analysing the pathogen-plant interactions as well as answer some basic questions concerning the interactions of the pathogen with cotton. Concurrently, we needed to find out what triggers the germination of *T. basicola* in the soil, which host interaction factors (signals) are involved in the process and whether fungal strains grow specifically towards their hosts. Since *T. basicola* shows biotrophic infection and it is an obligate pathogen, relying on its hosts to produce spores, we expected to find host-pathogen signalling possibly already at the stages of spore germination.

Signalling is most probably involved in host-pathogen recognition. To be able to infect, the pathogen must differentiate into infection structures and enter the root cells. In the longer term we aim at finding out what triggers the differentiation process of the fungus, first into infection structures and later on into its virulent stage and whether this process can be controlled.

Understanding the disease cycle, from its initiation and through to its completion is essential, including which factors influence different stages of the disease and whether these factors could be manipulated. An extensive literature survey (L. Pereg-Gerk and J. Baker, unpublished) led us to divide the disease cycle of black root rot into six major steps:
1) Germination of *T. basicola* spores in the soil.
2) Growth of the pathogen towards the plant roots.
3) Attachment to root surface: the first contact by the pathogen and host-pathogen recognition.
4) Differentiation of the pathogen into infection structures and penetration into the host cells.
5) Establishment of the biotrophic phase, a dormant disease stage.
6) Conversion to necrotrophy (root rotting), and the production of new spores. It is the black colour of the melanin coating the spores that gave the disease its name.

The plant and its pathogen would need to communicate in each step of the cycle for the disease to progress and for a "successful" completion of the final stage: the necrotrophic stage. Our future aim is to understand *T. basicola*-cotton communication (also refers to as pathogen-host signalling) and hopefully interfere with it to reduce disease.

Objectives

2. List the project objectives and the extent to which these have been achieved.

The main questions that we aim at investigating are (1) which is the crucial step in the infection process of cotton by *T. basicola*, leading to the disease, and how can it be controlled and (2) how does the plant responds to pathogenic *T. basicola* and how can this information be used to induce resistance. The objectives of this project were to produce tools that would enable us to investigate these questions.

More specifically our **objectives** as listed in the original research proposal submitted to the CRDC were:

Year 1: Objective: Optimise currently and previously developed systems for studying plant-pathogen interactions and complete the development of a transformation system for black root rot, with the **performance indicator** being: Reliable expression of a gene introduced into *T. basicola* (achieved).

Year 2: Objective: (1) Determine key factors controlling spore germination. (2) Demonstrate validity of research approach to identifying mechanisms of host specificity. (3) Screen fungal mutants, as well as cotton and non-cotton isolates of *T. basicola*, against host and non-host plants to identify combinations best suited for examining each critical stage of the infection process. The **performance indicators** were: (1) Wide range of host-strain combinations tested in repeated, replicated experiments, (2) Several mutant strains characterised and selected for use (both achieved).

Year 3: Objective: Find genetic factors affecting the interaction of *T. basicola* with its host, with the **performance indicator** being: Identify at least one gene that determines success of host-specific infection (five mutants were identified and the genes are under study).

Another **Milestone**, added in the progress report of September 2005: Establishment and optimisation of protein extraction methods for 2D protein analysis of *T. basicola* and that of cotton (*G. hirsutum*) root, with the **performance indicator** being: 2D protein reference maps established for several isolates of *T. basicola* obtained from different host plants and for cotton (*G. hirsutum*) roots (achieved).

During 2006 **two** other **objectives** were added, following development in proteomics: (1) To identify *T. basicola* proteins which are specifically expressed in presence of host root, and (2) To establish the relationship between host preference, virulence and *T. basicola* proteome (started in collaboration with J. Harvey, UQ) (work toward achieving both objectives is in progress and continues into the new project).

Methods

3. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.

The integrative research program within our group concentrates on the interactions between the pathogen and its cotton host (and, in another project by PhD candidate J. Moulynox, on the biocontrol of the disease). The researchers in our group employ a multidisciplinary approach to investigate molecular factors controlling these interactions. The project is targeted to find out what controls the crucial steps of cotton infection by *T. basicola,* and how this information could be utilised to reduce incidents leading to the black root rot disease.

Two main strategies have been developed by our group to study the *T. basicola* infection process:
1) Generation of *T. basicola* mutants, which are altered in the ability to complete one or more of the six steps in establishing infection. Such mutants can be used to elucidate genes and proteins responsible for its pathogenicity towards cotton (*Gossypium hirsutum*) and to the response of cotton to the pathogen.
2) Studying *T. basicola* isolates obtained from the field, which vary in their host range, to identify which stages in the infection process are blocked in non-host or resistant host plants.

Therefore, to achieve our goals, three main lines of investigation were developed:

- **Host specificity and pathogenicity determinants in natural *T. basicola* variants**

- **Molecular genetics:** Identification of fungal pathogenicity genes using random mutagenesis of *T. basicola* and analysis of mutants with reduced virulence.

- **Proteomics:** Analysis of *T. basicola* and cotton proteins involved in host-pathogen interactions (to elucidate host and pathogen interaction factors and host defence mechanisms).

The ideas of generating and analysing pathogenicity mutants in order to find virulence factors in the pathogen as well as to analyse changes in protein production under different conditions in order to identify interaction factors in both pathogen and plant were not novel as such. However, this type of work has never been done with *T. basicola* and cotton and very little has been done using such tools in other fungal-plant systems. Some methodologies were adopted from other systems, but had to be largely optimised for *T. basicola* and cotton, an involving process which has often been tedious and required special experience with handling plant-microbial interaction systems, molecular genetics and proteomics.

The major focus of this seed project has been to develop the necessary methodologies in order to have tools to investigate the interactions of the fungal pathogen with its hosts, in particular cotton.

In order to find factors that are involved in the induction of *T. basicola* germination, growth towards the host cotton plant and differentiation into a virulent form, it was desirable to produce mutants of the pathogen, that are impaired in these properties. A comparison of such mutant strains with wild-type *T. basicola* would enable us to find the genes and proteins which are responsible for the mutations, and hence to the respective phenotypes. However, **no molecular tools existed for *T. basicola* at the start of this project** and these had to be developed by L. Pereg-Gerk and M. Katz. Random mutagenesis was selected as a method to study the crucial steps in the infection process and their genetic background. For this purpose, an optimal transformation system, which would allow an insertion of a known and easily detectable element into the genome of the pathogen, *T. basicola*, needed to be developed. A random insertion of such element into the pathogen genome would create a bank of mutants, which then be analysed for their ability to interact with the host plant.

Upon development of the transformation system, random mutagenesis and screening of fungal mutants affected in host-specific infection have followed. The mutants of interest were those that showed modified patterns of interaction with the host plant (cotton) to that of wild-type *T. basicola*. In future projects, the gene/s responsible for the respective change in pathogenicity will be analysed, as well as the phenotypes controlled by the gene/s and how they may be regulated.

The following steps were required for the generation of random pathogenicity mutants of *T. basicola*:

- First step: development of a reliable genetic transformation system for the fungus
- Second step: development of mass-tests to screen for pathogenicity mutants
- Third step: development of verification techniques to confirm reduced pathogenicity in soil
- Forth step: analysis and characterisation of pathogenicity mutants (and genes)

The first step of the development of a method for random mutagenesis (integrative transformation) of *T. basicola*, based on PEG-mediated transformation techniques using fungal protoplasts, was the establishment of a protocol for the production of *T. basicola* protoplasts. The protoplasts (naked cells) were then used for the random insertion of a known DNA element into the fungal genome. Another method, based on *Agrobacterium tumefaciens*-mediated transformation, which does not require the production of protoplasts, is currently being tested.

Pathogenicity of *T. basicola* isolates and mutants as well as their growth towards plants had been estimated using the dipping technique, where cotton seedlings are dipped in a spore suspension and directional growth tests, where spores were allowed to germinate and grow on agar towards cotton. To confirm the random insertion of the genetic element into the fungal genome, a Southern Blot hybridisation assay had been conducted for which total fungal DNA needed to be produced (so, a method for total genomic DNA extraction had to be adopted and optimised). To confirm reduced pathogenicity of *T. basicola* transformants, soil assays were conducted under laboratory conditions.

Proteomics of *T. basicola*-cotton interactions

Proteomics tools have been developed in order to study gene expression or suppression in the pathogen in response to signalling from the host plant and *vice versa*. The overall goal is to use protein mapping (proteomics) to investigate the complex interaction between *T. basicola* and its host plants: cotton. A basic understanding of the molecular background of this interaction and its regulation at the cellular level can help in the development of effective fungal control substances that could be used in disease management or in cotton breeding towards disease resistance.

Under certain growth conditions some genes may not be active, resulting in the absence of certain proteins. This is why the proteome of an organism, such as fungi, is dynamic and protein presence may change depending on the growth conditions. Comparing whole protein maps of *T. basicola* cultures grown in the presence and absence of a host plant (e.g. cotton), can teach us about the proteins which are specifically produced in the pathogen in reaction to a signal from the host plant. Since each protein is encoded for by a specific gene, if we find *T. basicola* proteins which are produced only in the presence of cotton, we could also isolate the corresponding genes which are involved in the interaction of *T. basicola* with cotton. Such genes are probably required for *T. basicola* pathogenicity towards cotton and for causing black root rot.

Development of protein sample preparation procedures: Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) can separate thousands of proteins. The most critical step in 2-DE is the preparation of the sample. Because each sample is different both in term of proteins and interfering substances, the first step in the proteomics work was to determine the best combination of reagents and steps necessary to obtain a high-quality 2-DE gel for *T. basicola* and *G. hirsutum* root.

2-DE of *T. basicola:* At the beginning of this project, there was only very few 2-DE studies on filamentous fungi, it was therefore necessary to test protocols of different protein extraction methods. The effect of different chaotropes, different precipitation methods, different pH and different detergents were tested. It was found that an SDS extraction of the fungal proteins prior solubilisation in the isoelectrofocusing buffer was the best protocol to be used to obtain a high quality 2-DE of *T. basicola*.

2-DE of *G. hirsutum* root: Several papers reported protein extraction methods from plants. Several of these methods were tested. It was found that a phenol extraction followed by an ammonium acetate/methanol precipitation was required to obtain a high-quality 2-DE.

Protein reference maps of *T. basicola* and *G. hirsutum*: Protein reference maps of *T. basicola* cotton isolate grown in a rich medium was obtained according to published protocol in a pH 3-10 and 4-7 and 8-200 kDa range while a protein reference map for *G. hirsutum* root grown in hydroponic solution was obtained for a pH 4-7 and 8-200 kDa range.

The proteome reaction of *T. basicola* to cotton root extract: endoconidia prepared from *T. basicola* cotton isolate grown on ½ PDA plates. The endoconidia were grown in Czapek Dox medium and Czapek Dox medium supplemented with host and non-host root extract. Proteins were isolated and analysed by 2-DE.

Results

4. Detail and discuss the results for each objective including the statistical analysis of results.

Year 1: ***2004/05 :****Objective****: Optimise currently and previously developed systems for studying plant-pathogen interactions and complete the development of a transformation system for black root rot, with the performance indicator being: Reliable expression of a gene introduced into T. basicola (achieved).***

The CI, L. Pereg-Gerk, together with the postdoctoral fellow J. Baker, set up a laboratory specifically for molecular pathogen-cotton interaction work, including the establishment of a PC2 laboratory and obtaining OGTR approval. A PC2 hazardous cabinet and plant growth incubators were fitted into the PC2 laboratory for fungal and plant work. Microscopy equipment had been upgraded for this work and a digital camera added to allow documentation of the results. In 2005, equipment for 2D electrophoresis of protein gels was purchased using a University Research Grant obtained by the postdoctoral fellow J. Coumans for this project. It allowed the commencement of fungal and cotton root proteomics work.

A collection of *T. basicola* isolates from different hosts has been established as well as a collection of plant seeds for host specificity experiments (Tables 1 and 2).

Table 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*	Carrot BT	John Harvey, UQ
Lupin	JHA21	John Harvey, UQ
Pansy	03185	John Harvey, UQ

* *T. thielavioides*, all others were *T. basicola* isolates

Table 2. Plant seed collection

Plant	**Scientific name of plant**	**Cultivar**	**Obtained from**
Cotton	*Gossypium hirsutum*	SICOT 189 BR	NsW-DPI 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
Durum Wheat	*Triticum durum*	Wollaroi	UNE
Broccoli	*Brassica oleracea*	Green sprouting	Krempin's seeds
Rice	*Oryza sativa*	Jarrah	UNE

Genetic transformation and analysis of pathogenicity mutants has served as an efficient tool for the isolation of genes important for pathogenicity from several other fungal pathogens. We identified a transformation system suitable for *T. basicola* and established a protocol for a PEG-mediated transformation system for genetic manipulation of *T. basicola* (transformation protocols developed by M. Katz and L. Pereg-Gerk and work undertaken by S. Al-Jaaidi). Such a transformation system, which allows an insertion of a known and easily detectable element into the genome of the pathogen, enabled us to produce transformants of the pathogen, with random insertion of the plasmid in their genomes (Figure 2). Comparison of such mutant strains with wild-type *T. basicola* would enable us to find the genes and proteins which are responsible for the mutations and, hence, for the respective phenotypes. Our ability to grow the fungal transformants on media containing the antibiotic hygromycin as well as verification using Southern Blot hybridisation analysis, indicated we successfully introduced a gene (for hygromycin resistance) into *T. basicola* which was also successfully expressed.

We used the developed protocol to produce an initial bank of fungal putative mutants, which were analysed for their ability to interact with the host plant and cause disease. The plasmid DNA inserted into the fungal genome using PEG-mediate transformation randomly disrupts genes, some of which may be involved in *T. basicola* pathogenicity. We screened for mutants with reduced pathogenicity to be able to analyse the genes and proteins affected. Application of this technique is likely to provide knowledge on genes and proteins required for *T. basicola*-host interaction leading to black root rot and assist future studies to target resistance breeding in plants.

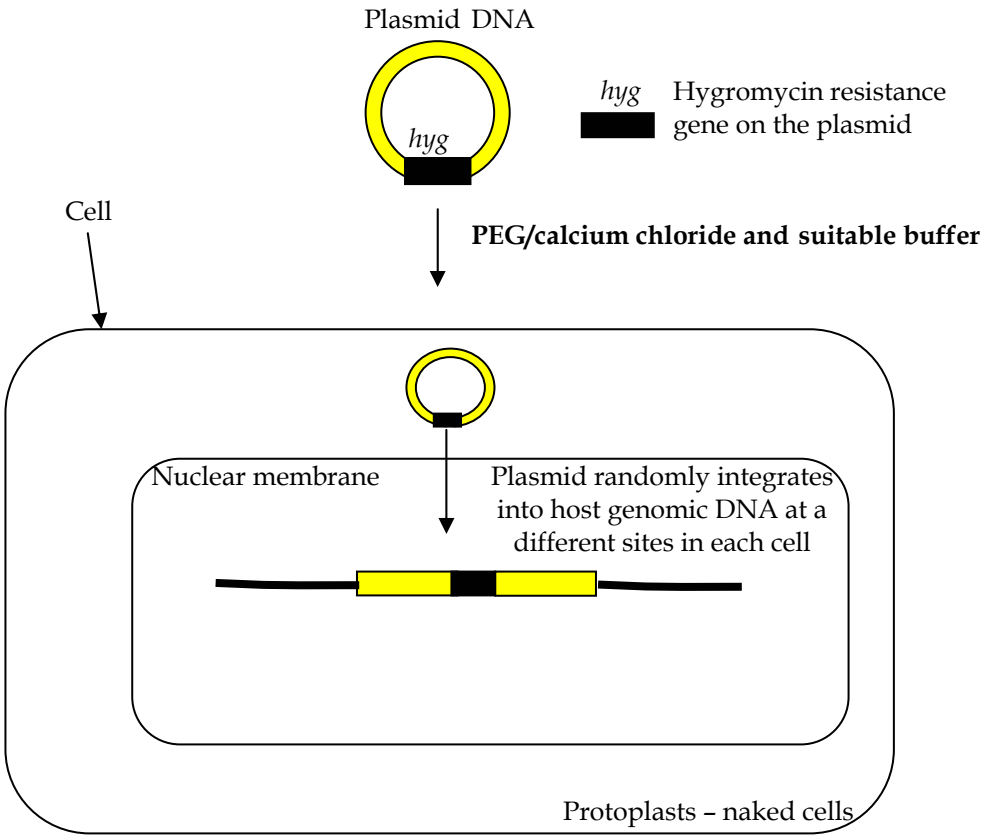

Figure 2. PEG-mediated transformation of fungal protoplasts. Random insertion of an entire plasmid into *T. basicola* genome, causing mutations.

The cell walls of fungal spores are often thick and rigid to allow the spore to survive in the soil under adverse conditions. As the genetic transformation of *T. basicola* requires the insertion of plasmid DNA into the fungal genome, the cell wall of the spores has to be removed leaving naked cells surrounded only by a cellular membrane, named protoplasts (Figure 3).

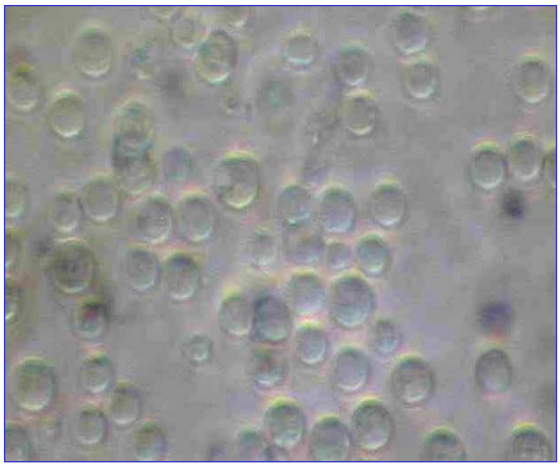

Figure 3. Protoplasts of *T. basicola*.
PEG-mediated transformation requires the production of protoplasts (naked cells, lacking cell wall) from spores, to allow plasmid uptake.

Many factors had to be considered and investigated and different conditions had to be tested to be able to produce protoplasts, including: the age of the fungal culture; the type of spores to be used; the age of the spores; which lysing enzyme; the right concentration of lysing enzyme; how long the incubation periods should be and under which temperatures; the concentration of PEG; how many washing steps and at which stages; whether to use fungal regeneration broth or solid regeneration medium; the type of regeneration broth or medium; the concentration of regeneration broth or medium and more.

Following the production of protoplasts, we inserted an integrative plasmid carrying the *hph* gene (hygromycin phosphotransferase) into the fungal genome. The *hph* gene provides resistance to the antibiotics hygromycin. Hygromycin resistance served as a selectable marker to identify those fungal cells that ended up with the plasmid in their genome (Figure 2).

We achieved transformation frequencies of 1.3-2.7 transformants per μg of plasmid DNA. Our ability to grow the fungal transformants on media containing the antibiotic hygromycin (100 μg/ml hygromycin B) indicated we successfully inserted a plasmid into the genome of *T. basicola*, producing fungal transformants and possibly causing functional mutations related to the infection process. Approx. 86% of the transformants showed mitotic stability. Southern hybridization analysis of genomic DNA from several fungal transformants, confirmed that hygromycin B resistance resulted from random integration of the plasmid into the fungal genome.

We have been using the PEG-mediated transformation protocol to produce hundreds of *T. basicola* transformants, to be screened for reduced pathogenicity against cotton hosts. Comparison of mutant strains, generated using the transformation protocol, with wild-type *T. basicola* would enable us to find the genes and proteins which are responsible for the mutations. The mutants of interest are those that show modified patterns of interaction with the host plant (cotton) to that of wild-type *T. basicola*.

Year 2: 2005/06 Objective: ***(1) Determine key factors controlling spore germination. (2) Demonstrate validity of research approach to identifying mechanisms of host specificity. (3) Screen fungal mutants, as well as cotton and non-cotton isolates of T. basicola, against host and non-host plants to identify combinations best suited for examining each critical stage of the infection process. The*** *performance indicators* ***were: (1) Wide range of host-strain combinations tested in repeated, replicated experiments, (2) Several mutant strains characterised and selected for use (both achieved).***

Optimal systems for **mass testing** of root infection and the extent of the black root rot disease have been developed. We conducted two assays: one to test plant infection by direct exposure of the germinating root to a fungal spore suspension (pathogenicity tests, Figure 4) and the other testing whether the growth of the fungal hyphae is directed towards the growing roots (directional growth tests, Figure 5). This system was designed to allow large scale experiments and enable testing the pathogenicity levels of a large number of both naturally occurring *T. basicola* strains and putative mutants towards cotton and other plants.

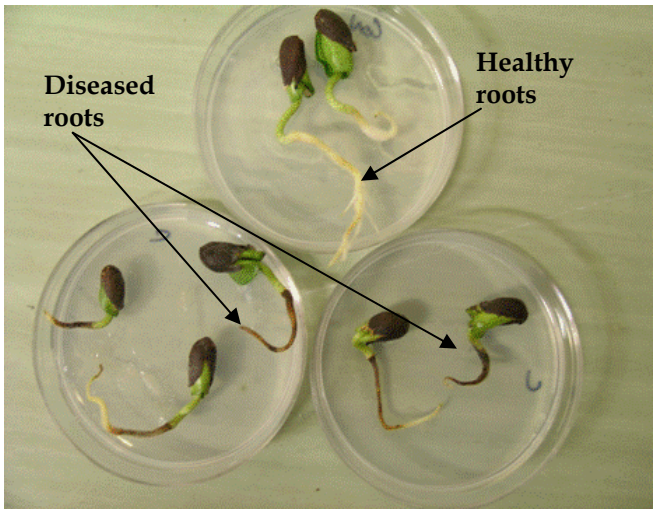

Figure 4. Pathogenicity tests on agar plates. Lower plates show seedlings inoculated by *T. basicola*, affected by black root rot. Upper plate shows control of disease-free, non-inoculated, cotton seedling.

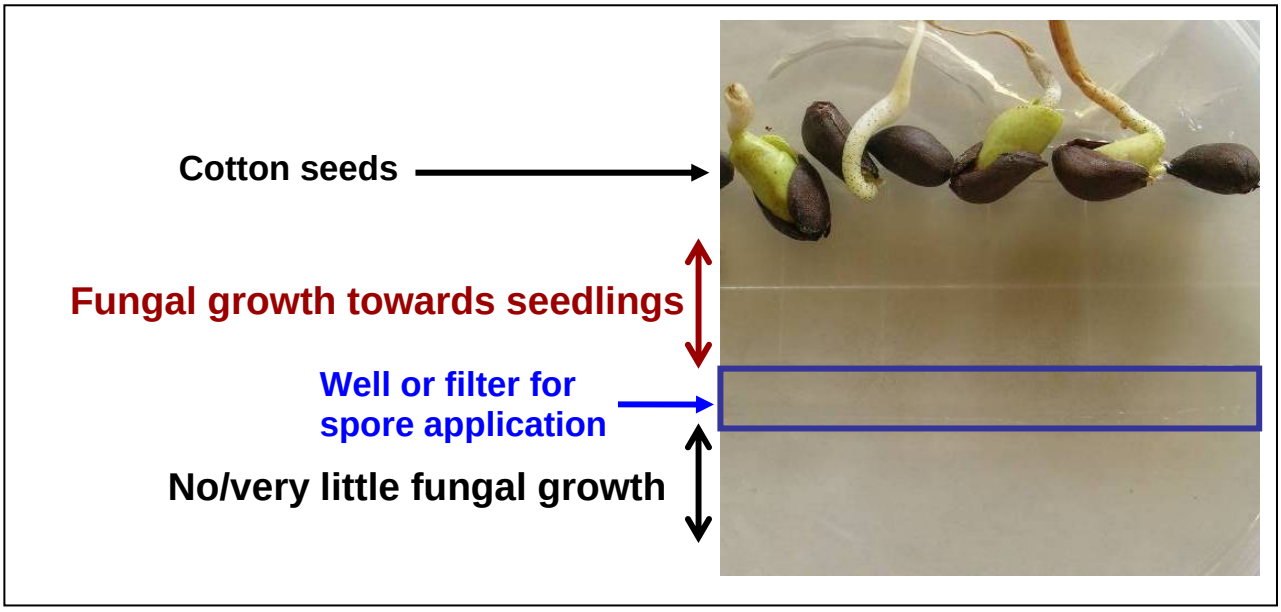

Figure 5. Directional growth of fungal hyphea towards cotton seedlings

Germination and growth of the fungi in the presence of host and non-host plants were tested on water agar plates. We performed pathogenicity tests and directional growth tests of different *T. basicola* strains towards host and non-host plants. In the absence of any plant there was no or very little germination under these conditions. The few germinated spores showed very little growths.

Pathogenicity tests

A total of seven *T. basicola* strains: one isolated from cotton (BRIP40192), two from lettuce, two from carrot, one from lupin and one from pansy (Table 1 above), were tested for pathogenicity towards seedlings of plants known to be affected by black root rot: cotton, lettuce, carrot, lupin and pansy, and as a control seedling of wheat, broccoli and rice (not known to develop black root rot).

Several growth systems were tested for their suitability to reliably examine pathogenicity of *T. basicola* strains toward plant seedlings. Six systems were tested and compared, however, the most reliable and consistent results were obtained using the dipping technique and the soil assays. Other techniques, such as using gnotobiotic chambers or using hydroponic growth systems, gave inconsistent results with most plants.

Host specificity was determined according to the severity of the disease on hosts infected with different *T. basicola* strains (Table 3). The severity of the disease varied between plants infected with different *T. basicola* strains (Figure 6), indicating pathogen-host specificity. E.g., cotton seedlings showed only slight signs of disease when inoculated with *T. basicola* strains isolated from lettuce or carrot but got highly diseased by *T. basicola* strains isolated from cotton, lupin and pansy. However, lupin was highly affected by all *T. basicola* strains except of the one isolated from pansy (Figure 6). Control seedlings, namely wheat, broccoli and rice, did not show any black root rot disease when infected with endoconidia of the different *T. basicola* strains.

It is interesting to note that wheat seedlings, although not showing signs of black root rot, did contain *T. basicola* spores in their roots (shown by former Honours student, G. Mijajlovic). Thus wheat is a host to the fungus but is not susceptible to the disease (avoiding the necrotrophic stage).

Table 3. Rating of necrotic lesions induced by *Thielaviopsis* isolates on roots of various plants

Necrotic root lesion (% from the total length of the root)	Determined susceptibility to *T. basicola* strain
0-20	Non-susceptible
21-40	Non-susceptible
41-60	Susceptible
61-100	Susceptible

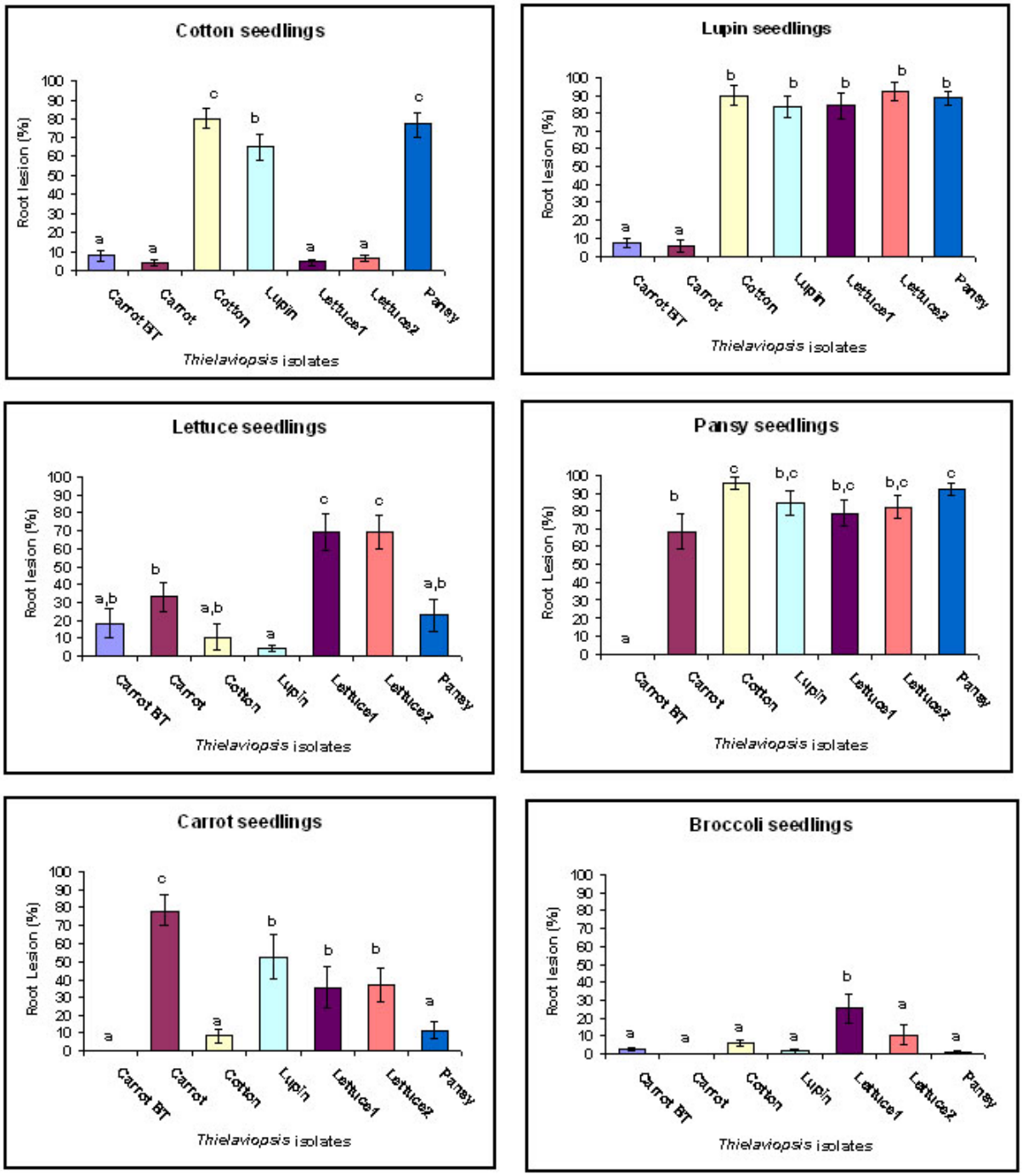

Figure 6. Pathogenicity of *Thielaviopsis* isolates towards seedlings of different plants. Lesion on roots were measured 7 days post inoculation with 3.5 x 10^5 spores/ml. Treatments within each box labeled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Bars represent standard errors of the mean from 3 separate experiments.

Directional growth tests

Although we could detect certain levels of host specificity in pathogenicity, directional growth of all the seven *T. basicola* strains towards all of the tested seedlings, whether they were hosts or not, was evident in a system containing one plant versus one *T. basicola* strain on each agar plate (Figure 7).

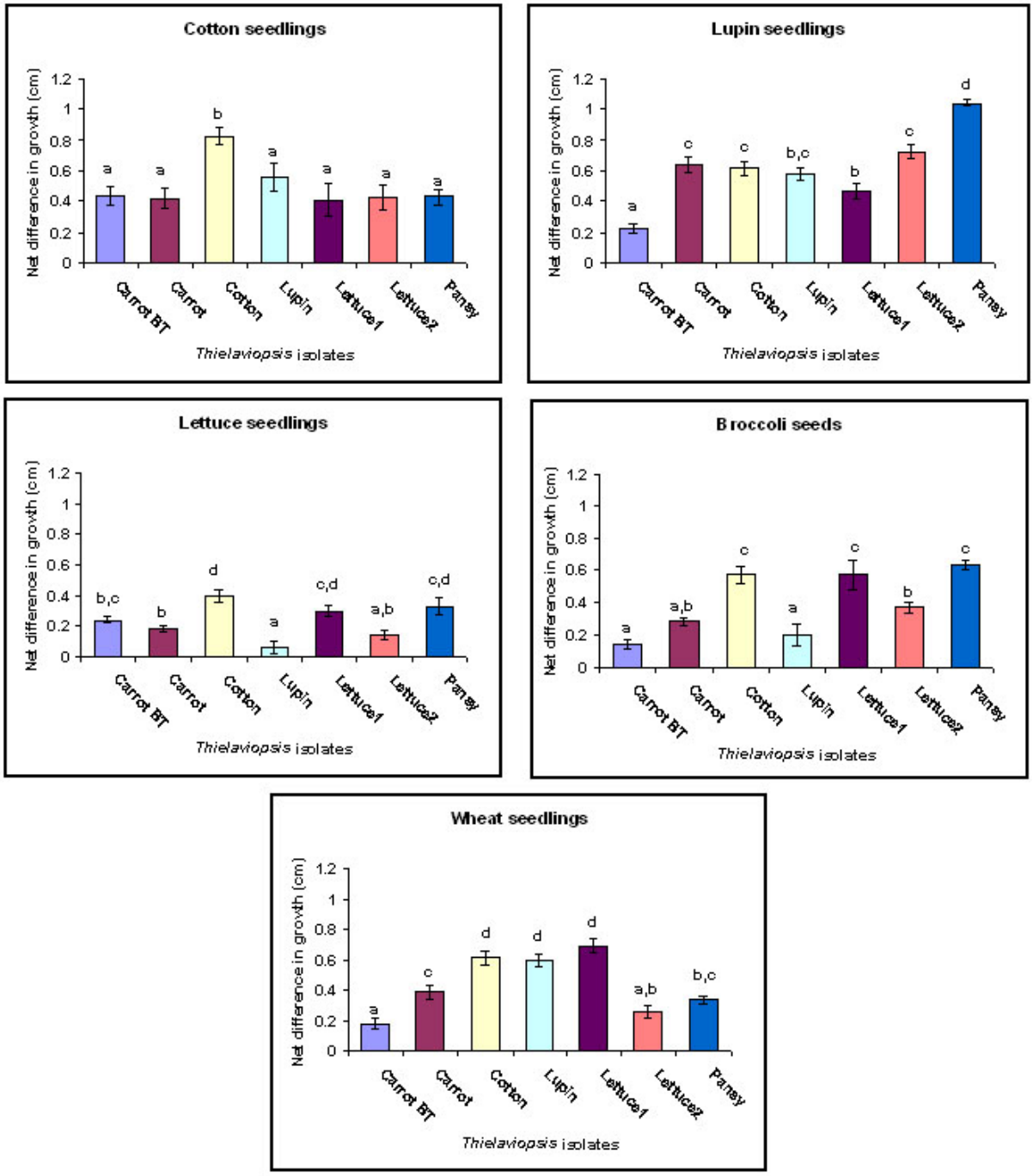

Figure 7. Directional growth of *Thielaviopsis* isolates towards seedlings. The data above shows net differences in growth (in cm) towards and away from germinating seeds, adjusted for relative growth rates of the isolates. Inoculum of 70 µl at a concentration of 3.5×10^5 endoconidia/ml was applied to filter paper strips and exposed to 4 seeds per plate. Growth of mycelia was measured seven days post inoculation. Columns within a plant species labeled 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.

In competition tests, exposing each *T. basicola* strain to the plant from which it was isolated on the one side and to another plant on the other side of an agar plate, many of the *T. basicola* strains showed preference of growth towards specific plants, **in particular cotton**, as shown in the three examples below:

Growth of cotton isolate of *T. basicola* towards cotton seeds Vs. 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

Growth of lupin isolate of *T. basicola* towards lupin seeds Vs. 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

Growth of lettuce 1 isolate of *T. basicola* towards lettuce seeds Vs. 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

Growth of the lupin and lettuce1 *T. basicola* isolates towards seeds (weight of seeds indicated)

T. 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

We briefly conclude that under lab conditions germination of *T. basicola* spores is most likely triggered by exudates from a large variety of plants and not only by its host. It is most likely responding to the presence of nutrients excreted by the seedling. However, there is some difference in the growth of the fungi towards different plants. We can see that germinating cotton seeds highly attract the different *T. basicola* strains, even those that were shown not to cause disease in cotton (such as the lettuce strains). We suggest that germinating cotton seeds excrete large amounts of exudates and/or signals that initiate germination of *T. basicola* spores and attract the fungus to grow specifically towards the direction of the seedlings.

We also conclude that *T. basicola* strains exhibit a degree of host-specificity. Plants can be divided into (1) non-hosts (not infected by *T. basicola*), (2) non-susceptible, or resistant, hosts (infected but do not exhibit disease signs) and (3) susceptible hosts (diseased upon infection). Cotton in Australia is highly susceptible to many, but not all, strains of *T. basicola*, and the Australian cotton industry is suffering from up to 40% yield loss due to direct and indirect effects of this pathogen. Our main objective in future work is to explain why some fungal-root interactions are compatible, leading to disease, and others are incompatible, leading to the phenomenon of resistance.

Production of T. basicola pathogenicity mutants using PEG-mediate transformation

A total of 202 putative transformants were screened for aggressiveness towards cotton (disease severity) and directional growth of germinated spores towards cotton. All putative transformants showed no defect in directional growth towards cotton seeds, however, five strains showed reduced pathogenicity towards cotton (Figure 8).

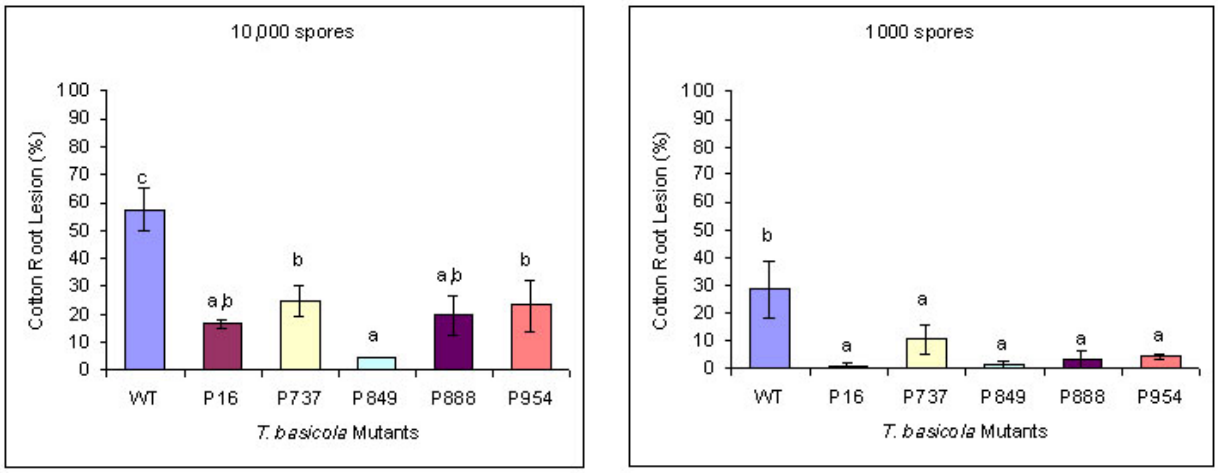

Figure 8. Disease severity of cotton seedlings inoculated with *T. basicola* pathogenicity mutants using the dipping technique. Percentage of lesion from total length of inoculated roots (dipped in a solution of 3.5 x 10^4 endoconidia/ml) was recorded 7 days post inoculation. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical bars represent standard errors based on 8 replicates.

Year 3: ***2006/07*** *Objective:* ***Find genetic factors affecting the interaction of T. basicola with its host, with the*** *performance indicator* ***being: Identify at least one gene that determines success of host-specific infection (five mutants were identified and the genes are under study).***

The five *T. basicola* mutants, which showed reduced pathogenicity towards cotton, showed reduced virulence also towards lupin using the dipping technique (Figure 9).

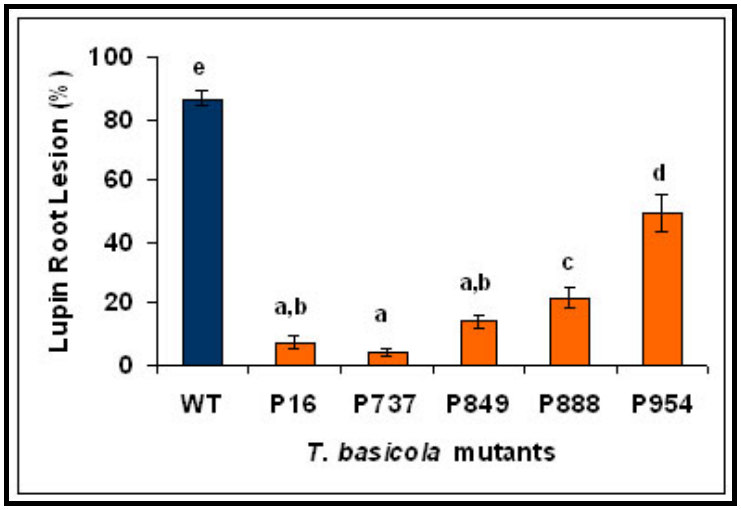

Figure 9. Disease severity of lupin seedlings inoculated with *T. basicola* pathogenicity mutants using the dipping technique. Percentage of lesion from total length of inoculated roots (dipped in a solution of 3.5 x 10^4 endoconidia/ml) was recorded 7 days post inoculation. Columns labelled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Vertical bars represent standard errors based on 8 replicates.

For confirmation, the five mutants were tested for pathogenicity towards cotton in soil (Figure 10, top photo) and were found to be less aggressive than the wild-type strain even at high inoculum levels (Figure 10, bottom). In the following project (CCC-CRC project 1.01.55, commenced mid August 2007), we will continue to optimise the transformation system, to produce hundreds of transformants, test for pathogenicity and search for further pathogenicity mutant. Once a collection of such mutants will be further established, the gene/s responsible for the reduced pathogenicity will be analysed, as well as the phenotypes it/they controls and how it may be regulated.

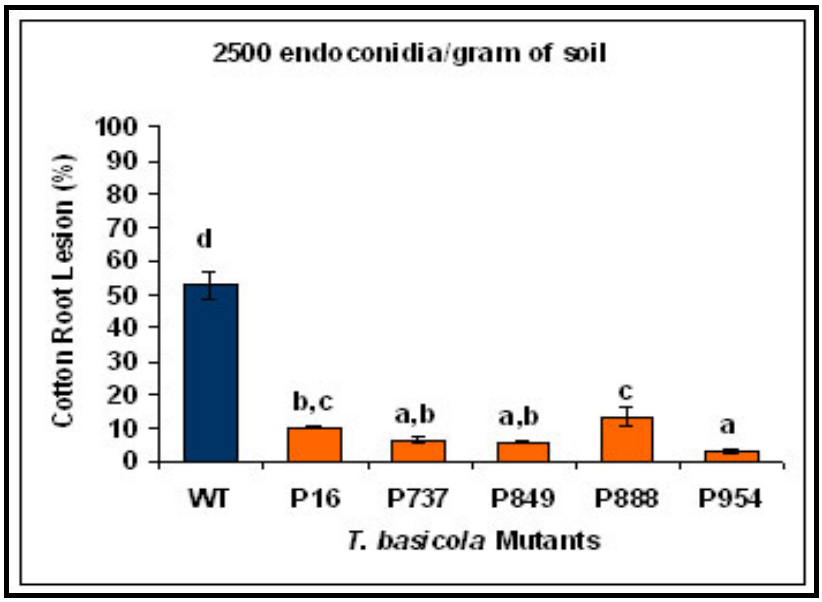

Figure 10. Disease severity on cotton seedlings inoculated with *T. basicola* pathogenicity mutants in soil assays. Percentage of lesion from total length of roots (grown in soil containing 2500 endoconidia/g soil – soil system is shown in the top photo) was recorded 10 days post inoculation. Negative 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 bars represent standard errors based on 30 replicates (from 3 experiments).

Initial characterisation of the mutants revealed that their growth on rich or poor nutrient media was not affected by the mutations (Figure 11) (so they are unlikely to have general metabolic mutations), nor were they affected in their growth patterns towards cotton roots (Figure 12). Therefore the mutations in these strains seems to more specific to their virulence towards cotton roots and thus relevant to the study of pathogen-cotton interactions. Other observations were the ranging ability of the mutants to produce melanin, a trait that have been implicated in fungal virulence in other systems and will be further analysed.

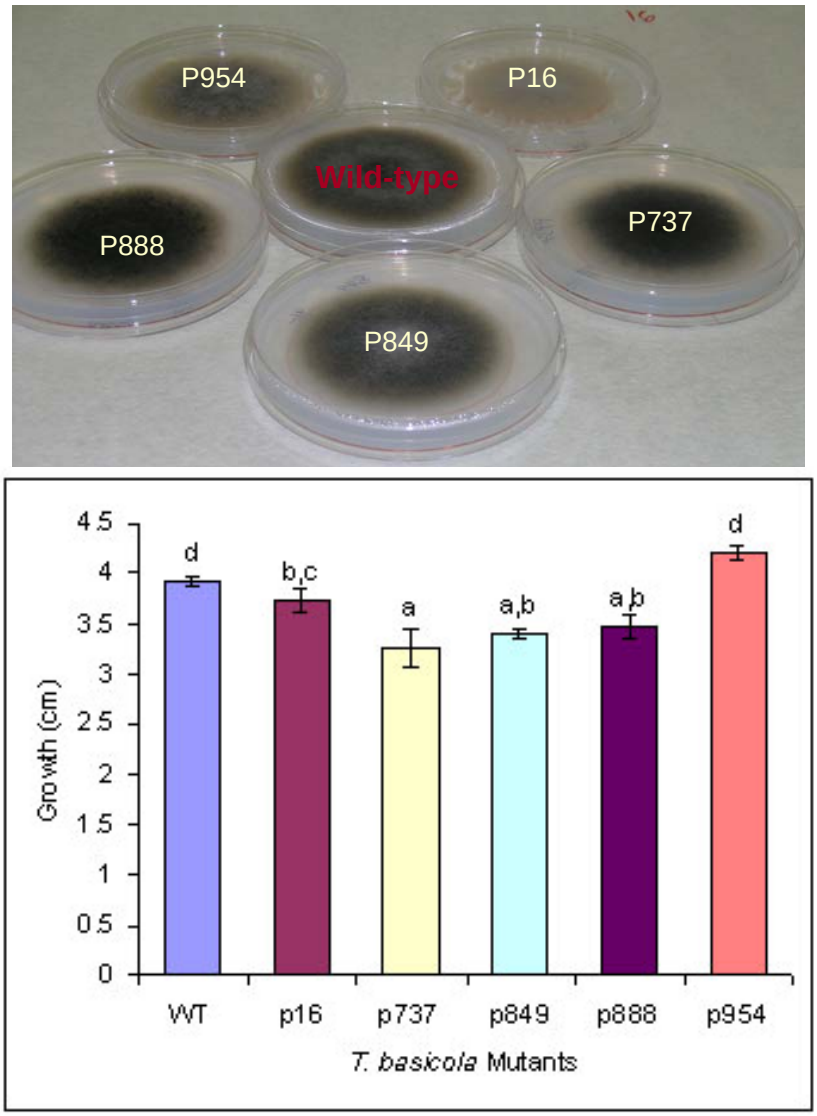

Figure11. Appearance of *T. basicola* pathogenicity mutants (top photo) and their growth in diameter (bottom figure) from the point of inoculation at the centre of each agar plate. Growth was measured in centimetres seven days after inoculation.

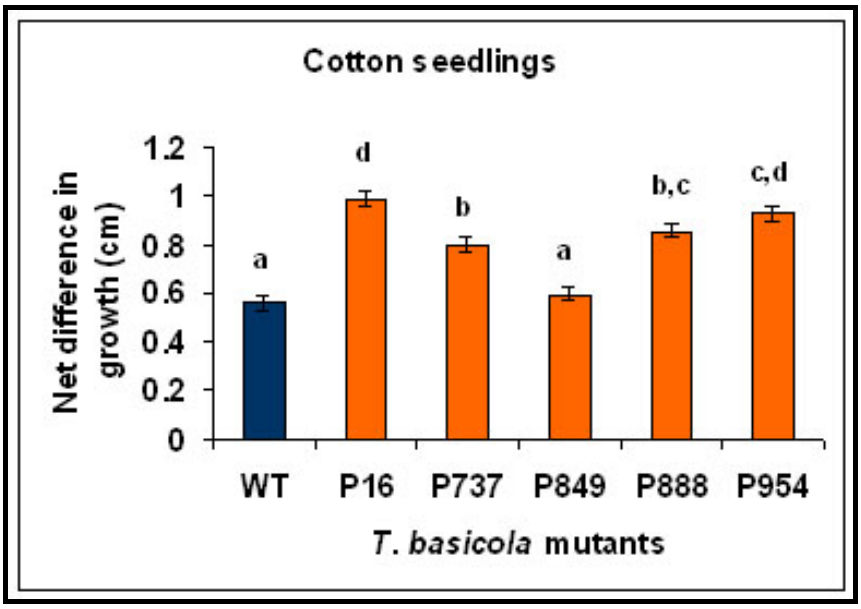

Figure 12. Directional growth of *T. basicola* pathogenicity mutants towards cotton seedlings. The data above shows net differences in growth (in cm) towards and away from germinating cotton seeds, adjusted for relative growth rates of mutant and wild-type *T. basiolca* strains. Inoculum of 70 µl at a concentration of 3.5 x 10^5 endoconidia/ml was applied to filter paper strips and exposed to 4 seeds per plate. Growth of mycelia was measured seven days post inoculation. Columns within a plant species labeled with the same letters do not differ significantly at $p < 0.05$ by Duncan's test. Bars represent standard errors of the mean from 5 replicates.

Another Milestone, added in the progress report of September 2005: Establishment and optimisation of protein extraction methods for 2D protein analysis of T. basicola and that of cotton (G. hirsutum) root, with the performance indicator being: 2D protein reference maps established for several isolates of T. basicola obtained from different host plants and for cotton (G. hirsutum) roots (achieved).

During 2006 two other objectives were added, following development in proteomics: (1) To identify T. basicola proteins which are specifically expressed in presence of host root, and (2) To establish the relationship between host preference, virulence and T. basicola proteome (started in collaboration with J. Harvey, UQ) (work toward achieving both objectives is in progress and continue into the new project).

Conditions to produce high quality protein extracts from *T. basicola* strains and from cotton roots for use in 2D protein gel electrophoresis analyses have been established and optimised. Total protein reference maps for different isolates of *T. basicola* from different host plants and for cotton roots are completed. A new extraction method for analysing the response of *T. basicola* to the presence of host and non-host plant is under development. Preliminary results were obtained from the comparison of different media.

Experimental design and the decision on research directions and experimental treatments used in this section were a result of group effort (J. Coumans, L. Pereg-Gerk, M. Katz and D. Backhouse), in particular at the earlier stages of the work. Protocols to handle and grow plants and *T. basicola* were provided by L. Pereg-Gerk. All protocols to extract cotton root and *T. basicola* proteins, to analyse these proteins and their use in 2D gel electrophoresis were developed/optimised by J. Coumans. Some experiments, as will be indicated below, were done in collaboration with John Harvey, UQ. The following Results section on proteomics was prepared by J. Coumans (pages 21-23).

Suitable protein extraction methods were obtained to produce high quality 2-DE of *T. basicola* grown in a rich medium and for *G. hirsutum* root (tap root and hypocotyl) grown in hydroponic solution (Fig 13 and 14).

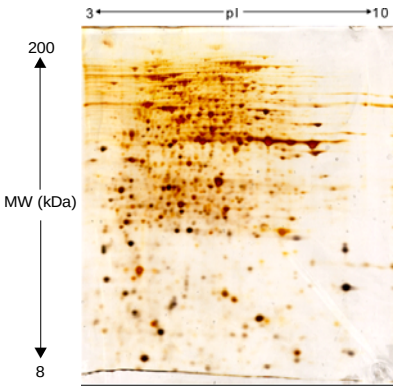

Fig 13: Silver staining of a 2-DE gel of *T. basicola* grown in PDB

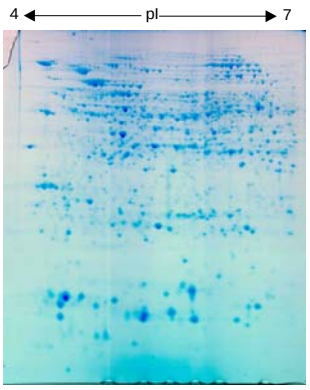

Fig 14: Blue silver staining of a 2-DE gel of cotton root

T. basicola endoconidia were grown in Czapek Dox media supplemented with root extract from two host plants (cotton and lupin) and two non-host plant (wheat and hairy vetch). Whole-cell proteins were extracted and samples were analysed by 2-DE (Fig 15). Visual examination of the 2-DE gels show visible differentially expressed protein spots. Quantitative expression analysis is underway.

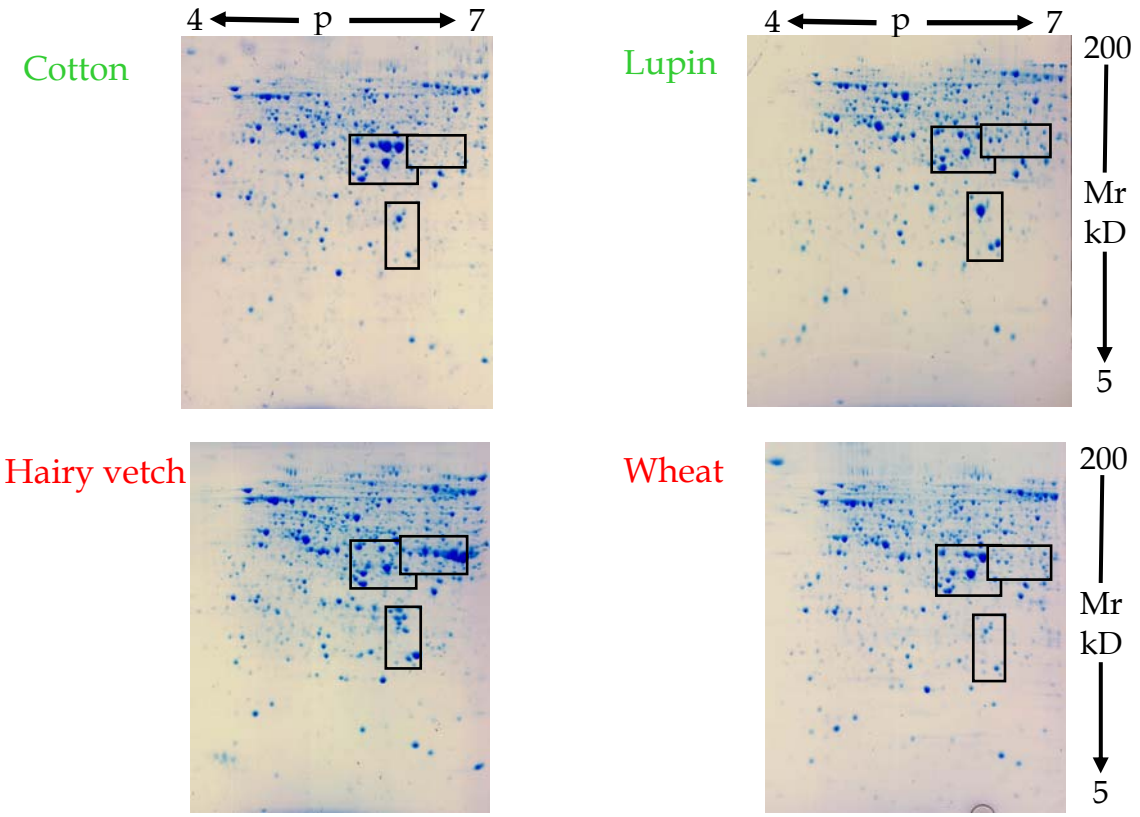

Fig 15: Blue silver staining of 2-DE gels of *T. basicola* grown in Czapek Dox medium supplemented with host (cotton, lupin) and non-host (hairy vetch and wheat) root extract. Black boxes highlight the proteins that show alterations in their expression pattern.

In order to evaluate the natural biodiversity in *T. basicola* and to see if a relation could be established between protein expressions, virulence towards cotton and preferred hosts, the proteomes of 12 different isolates retrieved from three different hosts has been studied. It was found that based on the protein expression level of more than 1000 protein spots, these isolates cluster according to the host from where they were retrieved (Fig 16).

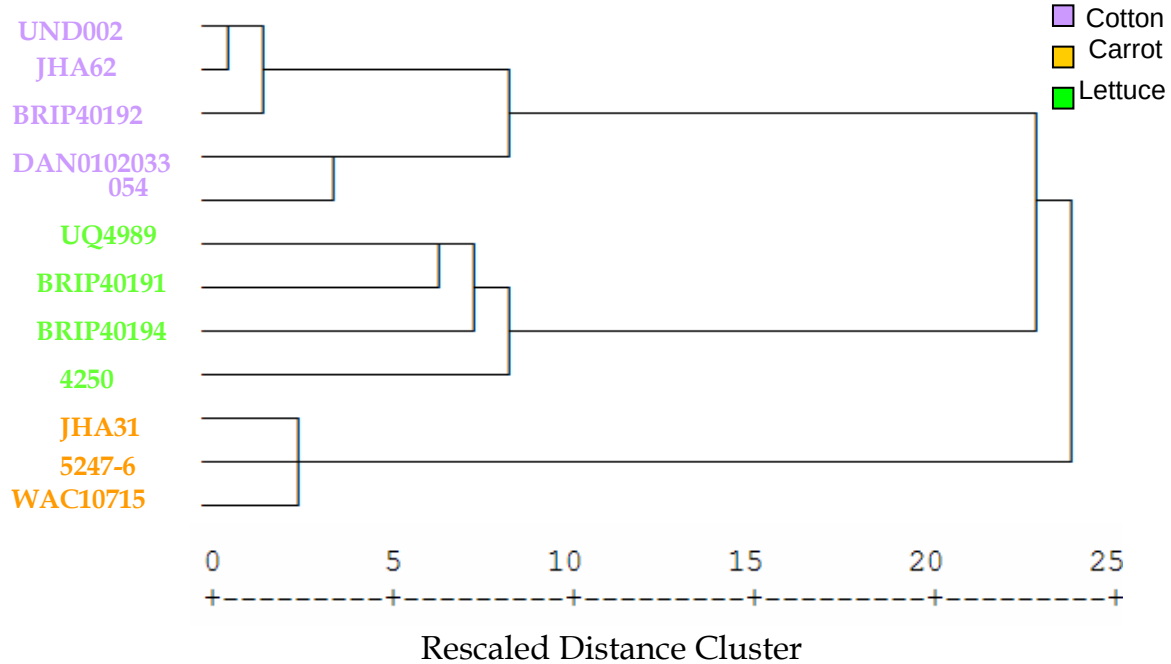

Fig 16: Classification of the proteomes from 12 isolates of *T. basicola*. Hierarchical clustering was performed using the Pearson correlation as metric and the nearest neighbour for linkage.

To identify potential virulence factors towards cotton, a spearman's correlation test between proteome results and pathogenicity results of J. Harvey (UQ) was performed. It was found that 9 and 43 protein spots correlated negatively at a level of significance of 0.01 and 0.05, respectively and that 5 and 25 protein spots correlated positively at a level of significance of 0.01 and 0.05 respectively with the pathogenicity results. Identification of these proteins by LC MS/MS is underway and will bring important information on the biological pathway involved in the plant recognition.

To analyse the proteome defence response of *G. hirsutum* through the interaction with *T. basicola,* a coordinated and reproducible infection of cotton root with *T. basicola* under controlled environmental conditions was developed. 3-days after inoculation, lesions characteristic of *T. basicola* infection were observed. After 7-days, most of the tap root presented the purplish-black discoloration characteristic of the disease and microscopic examination of the tap root confirmed that chlamydospores had formed on necrotic tissues.

Proteins were extracted from infected and non-inoculated (NI) roots and quantified using the 2-D quant kit from GE Healthcare Life Sciences. A slight increase in total protein content was observed one day after inoculation with *T. basicola* compared to non-inoculated cotton root follow by a steady decrease in protein concentration up to 7 day post inoculation (Fig 17).

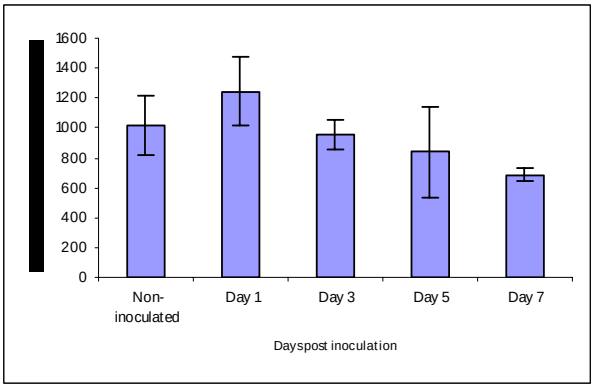

Fig 17. Quantity of protein per g of cotton root after inoculation with *T. basicola*. Error bars show the standard deviation (n=3).

To assess the global changes in the protein expression levels, proteins from NI, day 1, 3, 5 and 7 post-inoculation cotton root were analysed by 2-DE. Gels were compared and quantified using the PD-Quest software and the expression profiles of the protein spots were further analysed with Gene Cluster 3.0 and JavaTreeview software. Centred correlation was used as a measure of the distance between the different proteins spots while clustering was performed using the average linkage method. The relationship among the objects was visualised using the JavaTreeview software (Fig 18). This dendogram reveals 4 major protein expression profiles that were defined by taking groups of closely related proteins (coefficient correlation >0.8) and by calculating the average expression profile for these proteins. Protein spots that have an average expression volume twofold higher than those from NI samples will be identified by LC-MS/MS.

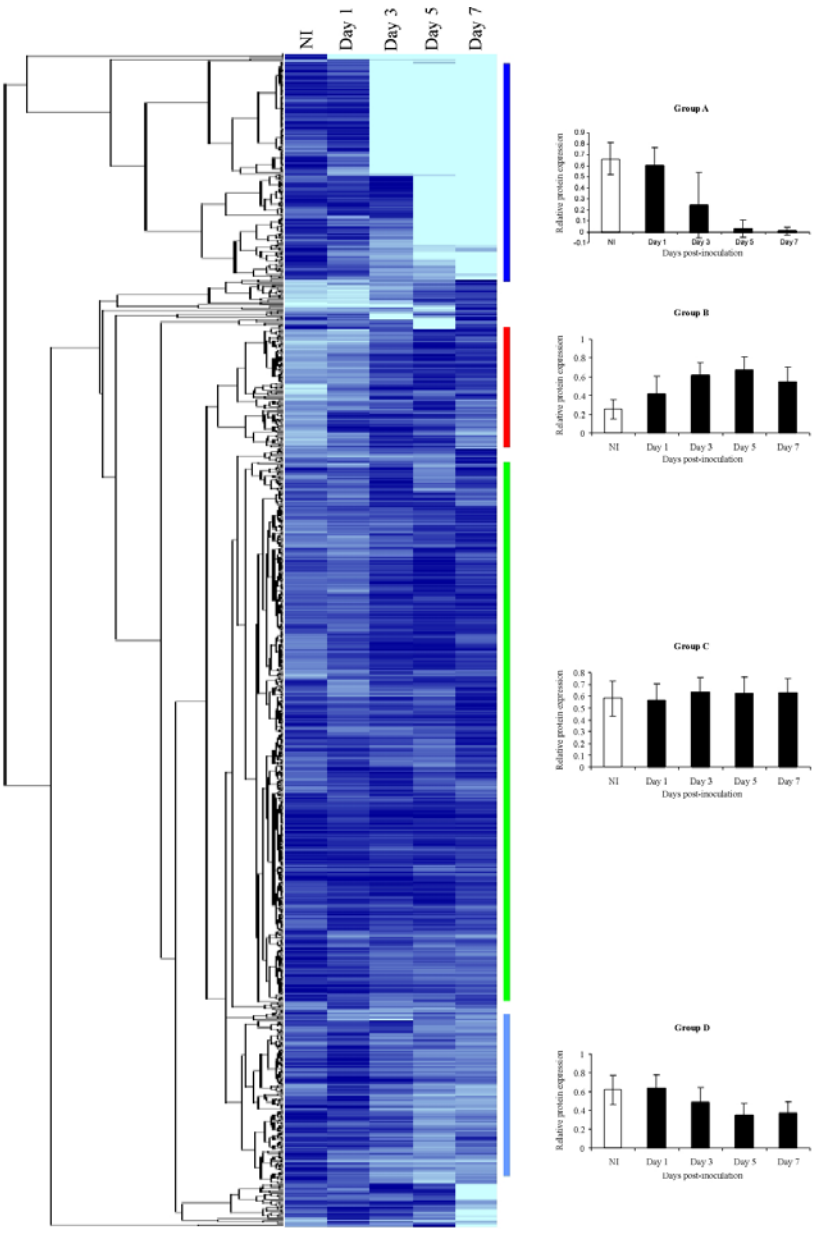

Fig 18. Cluster representation and expression profiles of protein expression in infected cotton root with *T. basicola*. Dendogram and blue-scale image were produced as described in the text. The colour scale range from clear blue for low percentage of expression to dark blue for high percentage of expression. Average expression profiles of the proteins included in the colour bands are show in right hand of Fig 18. Profiles were defined by taking groups of closely related proteins (correlation coefficient >0.8) and by calculating the average expression profile for these proteins. About 90% of the proteins were used to define these profiles.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

In the original project application (for 2004/5) it was requested to identify **Objective**, **Milestones** and **Performance indicators**, but not to list the outcomes (which are already included in the above categories). The way this report is organised is by addressing each objective and performance indicator, thus it already describes the planned outcomes achieved to date.

All of the objectives listed in the original application were achieved. As this seed project was mainly aimed at the production of research tools, most of its outcomes are tools (materials and methodology in fungal-plant interactions, molecular genetics and proteomics) which will be adopted by researchers in future and present projects targeting better understanding and control of black root rot and other cotton diseases.

The following list summarises the planned outcomes achieved in the project and how they will be adopted in future research:

Year 1: Objective: Optimise currently and previously developed systems for studying plant-pathogen interactions and complete the development of a transformation system for black root rot.
Milestones: Identification of a transformation system most suitable for *T. basicola*.
Performance indicator: Reliable expression of a gene introduced into *T. basicola*.

Year 1 objectives were achieved. A transformation system for *T. basicola* has developed and reliable expression of a hygromycin resistance gene introduced into its genome was achieved. Adoption in future research includes the use of the system to generate a mutant bank of *T. basicola* strains affected in their interactions with cotton, but otherwise metabolically normal. Such mutants will be studied in order to isolate pathogenicity related genes and investigate their role in pathogencity and how they could be controlled to reduce virulence.

Year 2: Objective: (1) Determine key factors controlling spore germination. (2) Demonstrate validity of research approach to identifying mechanisms of host specificity. (3) Screen fungal mutants, as well as cotton and non-cotton isolates of *T. basicola*, against host and non-host plants to identify combinations best suited for examining each critical stage of the infection process.
Milestones: (1) Testing the hypothesis that spore germination is specifically triggered by host factors. (2) Selection of fungal strains from a collection of fungal mutants for studying germination, infection and transition to pathogenic growth.
Performance indicator: (1) Wide range of host-strain combinations tested in repeated, replicated experiments.
(2) Several mutant strains characterised and selected for use.

Year 2 objectives were achieved. Methods were developed and optimised for the mass testing of genetically transformed *T. basicola* strain for the identification of mutants with reduced pathogenicity. Such methods will be applied in future research for the selection of pathogenicity mutants of interest. Four main stages of the pathogenicity cycle of *T. basicola* can now be tested for – its germination in response to plant exudates, its growth towards the plant seeds and seedlings, root colonisation and the level of virulence (reproduction and necrotrophy).

Naturally existing *T. basicola* strains isolated from different plants were studied and showed some degree of host specificity. Three levels of *T. basicola*-plant interactions were identified: plants can be divided into non-hosts (not infected by *T. basicola*), non-susceptible, or

resistant, hosts (infected but do not exhibit disease signs) and susceptible hosts (diseased upon infection). Cotton is a susceptible host to most strains of *T. basicola*, but is not susceptible to the *T. basicola* strain isolated from lettuce. Such strain can be used in future research, including in genetics and proteomics studies, to try and identify resistance mechanisms in cotton, by following the stage in which infection by this strain is blocked in cotton.

Germination of *T. basicola* and its growth towards the seed/seedling was triggered by susceptible hosts, non-susceptible hosts and non-host plant exudates. Cotton was found to be particularly attractive to a large selection of *T. basicola* strains (both virulent and avirulent towards cotton). What makes cotton exudates so attractive to *T. basicola* is the topic of a new grant application (eoI) submitted recently to the CRDC by L. Pereg-Gerk and D. Tucker.

Year 3: Objective: Find genetic factors affecting the interaction of *T. basicola* with its host.
Milestones: Isolate the affected genes from fungal mutants impaired in different stages of the disease.
Performance indicator: Identify at least one gene that determines success of host-specific infection.

Year 3 objective is an ongoing one as we are intending to produce a large bank of pathogenicity mutants and study the genes/proteins involved in the fungal virulence towards cotton. The methodology for analysing the mutants has been developed and used for the analysis of mutant properties and, currently, for the analysis of the pathogenicity gene/s affected. This part of the project is entering the second stage (in the new CCC-CRC project 1.01.55) – mass production of mutants using established methodology (developed in 1.01.21).

Another Milestone, added in the progress report of **September 2005**: Establishment and optimisation of protein extraction methods for 2D protein analysis of *T. basicola* and that of cotton (*G. hirsutum*) root, with the **performance indicator** being: 2D protein reference maps established for several isolates of *T. basicola* obtained from different host plants and for cotton (*G. hirsutum*) roots (achieved).

During 2006 two other objectives were added, following development in proteomics: (1) To identify *T. basicola* proteins which are specifically expressed in presence of host root, and (2) To establish the relationship between host preference, virulence and *T. basicola* proteome (started in collaboration with J. Harvey, UQ) (work toward achieving both objectives is in progress and continue into the new project).

Proteomics objectives have been achieved. Protocols for protein extraction methods for 2D-EG analysis have been established for both *T. basicola* and cotton roots. Reference protein maps were completed for both the pathogen and the cotton roots. Assays were developed to test the proteome reaction of cotton roots to the presence of the plant pathogen and to test the pathogen reaction to its host. Preliminary results have already been reported above. All of the tools developed in the seed project 1.01.21 are currently in use in the successive project 1.01.55 and have potential to be used in future projects on *T. basicola*–cotton interactions.

6. Please describe any:-
   a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);

b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and
c) required changes to the Intellectual Property register.

Currently, it does not seem necessary to make changes to the Intellectual Property register.

The projects in our group target black root rot, a seedling disease caused by *Thielaviopsis basicola*, which is a significant threat to cotton, especially in cooler areas and seasons. The genomics and proteomics projects aim to identify key molecular interactions between *T. basicola* and cotton roots and their control, to determine whether such interactions could be exploited in disease management. We aim at identifying genes and proteins responsible for pathogenicity, especially those involved in host-specific interactions. For example, identification of host-specific stimuli to pathogen differentiation into infectious form could be exploited by application of artificial germination stimulants or selection of cotton varieties with reduced stimulatory effect. Identification of host-specific interactions during infection could be used to find components of resistance that will increase the efficiency of breeding varieties with enhanced resistance.

Molecular tools (genomics and proteomics) required in the current project undertaken by our group (CCC-CRC project 1.01.55; 2007/8-2010/11) have been developed in the seed project (1.1.21), which was concluded in June 2007. Relevant establishments include: (1) a collection of *T. basicola* strains with confirmed degrees of virulence towards cotton, (2) a collection of plants tested and grouped into susceptible hosts, non-susceptible hosts and non-host for each of the *T. basicola* strains, (3) pathogenicity tests for large scale experiments and confirmation tests in soil, (4) a working procedure to produce *T. basicola* pathogenicity mutants, using a genetic transformation system, which allows an insertion of a known and easily detectable element into the genome of the pathogen. Currently we are in the process of testing an additional transformation method. (5) Tools for proteome analyses of both *T. basicola* and cotton, including optimisation of the techniques (the development of proteins extractions methods for the proteome study of *T. basicola* and *G. hirsutum* root and the establishment of reference maps for *T. basicola* and *G. hirsutum* roots).

There is no commercial IP in the above developments as these are based on published techniques, which were modified and optimised for use with cotton and with *T. basicola*.

The potential outcome to be commercialised are a gene or a suite of genes/proteins which confer resistance or assist in resistance of cotton to black root rot or soil additives to suppress the disease. IP that could lead to commercial value would most probably be based on strategies for genetic manipulation of cotton and/or on the nature of the soil additives.

Control mechanisms of pathogenicity could possibly be applied to the development of management strategies. Cotton breeders would gain knowledge on breeding towards reducing the plant attraction to the fungal pathogen and/or towards inducing defence mechanisms in cotton. In addition, farmers would be advised on new products for application in disease management.

In summary, possible IP in the future –

1. A set of pathogenicity related genes and/or proteins, essential for *T. basicola* virulence towards cotton/plants, identified.
2. Control mechanisms of pathogenicity gene expression or pathogenicity protein activity identified.
3. Measures for controlling pathogenicity related fungal genes/proteins and their use in plant breeding /soil amendments against *T. basicola* virulence developed. Soil amendments would be developed according to the pathogenicity genes/proteins

identified and their control measures. Plant breeding could then be towards reduced production of signals which enhance fungal virulence or towards increased production of substances which suppress fungal virulence.

4. Plant proteins involved in disease suppression could be identified and be utilised in plant breeding against black root rot and possibly other diseases.
5. Since work on pathogenicity genes of other fungi exist, the idea of utilising such information for disease suppression/resistance is not novel. It is novel for our system concerning *T. basicola* pathogenicity against cotton.

Conclusion

7. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

The black root rot fungus occurs as strains that are specific to particular host plants, and must establish a special relationship with living cells of the host root before root rotting can occur. This suggests that there are highly specific biochemical interactions between the fungus and cotton that are involved in the progress of the infection process.

The establishment of a large number of research tools in this project allows researchers to further investigate the complex interactions of *T. basicola* with cotton and to progress towards achieving a long term objective of the industry – to reduce the impact of the black root rot disease (and potentially other diseases) on cotton yields.

The tools established in the project would allow researchers to investigate whether there are host-specific triggers to certain stages of the pathogen infection and whether it is feasible to exploit key steps in the infection process for the induction of disease resistance in cotton or develop other control measures, such as soil amendments. Although *T. basicola* has a broad host range, individual strains are only capable of infecting, or causing disease symptoms in, a limited number of host species. This indicates the widespread occurrence of resistance mechanisms in plants, including cotton, that are effective against most strains of the fungus and which are presumably based on recognition events at the molecular level. Identifying the molecular basis of these mechanisms may enable them to be manipulated to increase the resistance of cotton to all strains of the fungus.

In addition, valuable information on the interactions of cotton with virulent and non-virulent strains of *T. basicola* was gathered in this project and is already used in a successive research to analyse cotton proteome response to its pathogen. The ability to produce *T. basicola* pathogenicity mutants and to study the proteome of cotton roots will enable us to use proteomics to identify proteins with altered expression in *T. basicola* mutants. Such proteins are most likely be involved in the response of the fungus to infection by the pathogen.

Another important outcome is the finding that cotton is highly attractive to both virulent and non-virulent *T. basicola* strains. It seems to be more attractive to the different pathogen strains than other plants and it is important to understand why this is the case.

The “take home message” is that a large number of modern and basic research tools have been established and the knowledge base on cotton interaction with *T. basicola* has been increased. Both developments allow researchers in our group to continue from the seed project into a full scale project towards understanding and battling black root rot.

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:
   (a) to further develop or to exploit the project technology.

(b) for the future presentation and dissemination of the project outcomes.
(c) for future research.

Technology and methodology established in this project can be further developed and exploited in future research towards industry priority areas. In the 2003-2008 annual operating plan of the CRDC, the fungal diseases Fusarium Wilt and Black Root Rot were identified as high priority risks to the cotton industry and black root rot still remains a risk. A current research project, CCC-CRC 1.01.55, based on the outcomes of the seed project, CCC-CRC 1.01.21, has strong links to Outputs 1 (Economic) and 2 (Environmental) of the CRDC by its potential of improving management of the cotton disease black root rot to reduce loss of yield, in a sustainable manner, with reduced input of fungicides and damage to the environment. The above corresponds well with the objectives of Program 3 of the CRDC. Thus, the most obvious contribution of the developments in the seed projects to CRDC strategies would be to crop protection via present and future research into the interactions of *T. basicola* with cotton.

There are 6-7 papers expected to be published in international, peer reviewed, scientific journals in the next 1-2 years. These are listed below in the next section of this report. Other publications would arise from the current subsequent project (1.01.55). With the assistance of the CCC-CRC and CRDC extension groups we hope to communicate with farmers and other industry partners and publish in industry newspapers and magazines (e.g. Spotlight, Cotton Grower). The concepts in a molecular genetics and proteomics type research are difficult to convey to non-experts, therefore we need the assistance of the extension groups to bring our messages to the cotton growers. We plan to keep on presenting in industry workshops such as FUSCOM and expert workshops and in the Cotton Conferences, as we did during the term of the seed project (see list of publications below). We will keep on presenting and keep our groups informed on recent development in relevant research fields by attending international and domestic scientific conferences.

Knowledge produced in current and future projects, using tools developed in the seed project, will be used by researchers and cotton grower groups in the production of cotton cultivars more resistant to black root rot and/or in the development of new black root rot management strategies, using soil microbe additives, or enhancing indigenous microflora. Both strategies will aim at reduction of cotton attractants to *T. basicola* in the soil at the seedling stages.

Project outcomes, including the development of protein extractions methods for the proteome study of *T. basicola* and *G. hirsutum* root and the establishment of reference maps for *T. basicola* and *G. hirsutum* root will allow future research to (1) investigate and potentially identify steps in the biological pathway used by *T. basicola* to grow in the presence of host and non-host plant; this will potentially lead to the use of natural or synthetic substances for germination of *T. basicola* spores in the absence of host plant, as well as to (2) the biochemical characterization of *T. basicola* isolates and finally to (3) potentially, the understanding of the inducible molecular defence mechanism of the cotton plant against *T. basicola* infection with the potential of using such information in designing protective strategies such as over expression of defence genes.

Identification of proteins of interest could not be achieved in the timeframe of the seed project, however, this project is ongoing and it is expected that the proteomics research will lead to three publications in international peer review journal in 2008 and possibly to future development of a *G. hirsutium* root proteome website.

8. A. List the publications arising from the research project and/or a publication plan.
(NB: Where possible, please provide a copy of any publication/s)

Publication plan for scientific journal articles

1. Pereg-Gerk, Baker, et al. Critical steps in *T. basicola* life cycle and its interaction with plants – a review. Possible journal: *Plant Pathology, Annual reviews in Microbiology* or similar.
2. Pereg-Gerk, Baker and Mijajlovic. Experimental factors in determining specificity in *T. basicola*-plant interactions leading to black root rot. Possible journal: *Plant Pathology* or similar.
3. Al-Jaaidi, Katz and Pereg-Gerk. Genetic transformation of the filamentous fungal pathogen *T. basicola*. Possible journal: *Molecular Microbiology* or similar.
4. Pereg-Gerk, Al-Jaaidi, Katz and Backhouse. Stages of *T. basicola* life cycle determining host specificity and virulence. Possible journal: *Plant Pathology* or similar.
5. Coumans, et al. Differentially expressed proteins in the interactions of *Thielaviopsis basicola* with root extract from the host and non-host plant. Possible journal: *Proteomics* or similar
6. Coumans, et al. Proteomic and genomic analysis of the diversity of 12 isolates of *Thielaviopsis basicola.* Possible journal: *Molecular and cellular biology* or similar (will depend what results from J. Harvey could be published)
7. Coumans, et al. Analysis of cotton (*Gossypium hirsutum*) root proteomes during a compatible interaction with *Thielaviopsis basicola*. Possible journal: *Proteomics* or similar

Professional reports

L. Pereg-Gerk, S. Al-Jaaidi, J. Coumans, J. Moulynox, M. Katz, D. Backhouse and D. Nehl (2006) "Molecular factors in pathogen-cotton interactions leading to Black Root Rot and disease control" Proceeding of *13th Australian Cotton Conference*, Gold Coast, August 2006.

Conference proceedings

1. Pereg-Gerk L., Al-Jaaidi S. and Katz M. (2007) Molecular factors involved in *Thielaviopsis basicola* -plant interactions. Genetic Society of Australia 54th Annual conference, Sydney, July 2007.
2. Pereg-Gerk, L., Coumans-Moens, J, Katz M., . Backhouse, D., and Al-Jaaidi, S. (2007) Molecular factors involved in *Thielaviopsis basicola*-plant interactions leading to black root rot. CCC-CRC Science Forum, Cotton Collective Week, Narrabri, August 2007 (presented by J. Moulynox for L. Pereg-Gerk).
3. Pereg-Gerk L., Katz M., Backhouse D., Al-Jaaidi S. and Forbes R. (2007) Molecular aspects of *Thielaviopsis basicola* Cotton-Interactions leading to Black Root Rot. FUSCOM workshop, Narrabri, 2007.
4. Coumans J. (2007) Proteomic Analysis of Black Root Rot of Cotton. FUSCOM workshop, Narrabri, 2007.
5. Pereg-Gerk, L. (2006) "Research summary: Molecular tools for the study of the cotton fungal pathogen T*hielaviopsis basicola*", *CoSERG meeting*, Sydney, September 2006.

6. Pereg-Gerk l., Al-Jaaidi S., Katz M. and Backhouse D. (2006) Molecular analysis of *Thielaviopsis basicola* Cotton-interactions leading to Black Root Rot. 13th Australian Cotton Conference, Gold Coast, Australia, August 2006.

7. Coumans J., Adams M. and Pereg-Gerk L. (2006) Proteomics reveals the adaptation of *Thielaviopsis basicola* to plants species. 13th Australian Cotton Conference, Gold Coast, Australia, August 2006.

8. Coumans J., Pereg-Gerk L., Aitken E.A.B, Nehl D.B, Harvey J.A (2006) Proteomic and genetic investigation of *Thielaviopsis basicola* isolates exhibiting different levels of pathogenicity towards cotton (*Gossypium hirsutum*). 11th Proteomics Symposium, Lorne, 3rd -5th Feb.

9. Coumans J., D. Backhouse, M. Katz and L. Pereg Gerk (2005) On the road towards a proteome analysis of *Thielaviopsis basicola* interactions with cotton root. FUSCOM Workshop, ACRI, Narrabri 1st-2nd June

10. Al-Jaaidi S., M. Katz, D. Backhouse and L. Pereg Gerk (2005) Transformation of *Thielaviopsis basicola*: A tool to study the host-pathogen interaction at the molecular level. FUSCOM Workshop, ACRI, Narrabri 1st-2nd June.

11. Pereg-Gerk, L., Margaret Katz, David Backhouse and Samiya Al-Jaaidi (2004) Interactions of the soil pathogen *Thielaviopsis basicola* with plants. Australian Society for Microbiology (ASM) National Conference, Sydney SuperDome, 26th sept-1st Oct.

12. Pereg Gerk, L., M. Katz, D. Backhouse and J. Baker (2004) *Thielaviopsis basicola* cotton-interactions leading to black root rot: A molecular approach. 12th Australian Cotton Conference, Gold Coast, August 10th-12th.

13. Al-Jaaidi S., M. Katz, D. Backhouse and L. Pereg Gerk (2004) Transformation of *Thielaviopsis basicola*: A tool to study the host-pathogen interaction at the molecular level. Genetic Society of Australia 51st Annual conference, Melbourne, July 11th-14th.

14. Pereg Gerk, L., J. Baker, S. Al-Jaaidi, M. Katz, D. Backhouse and D. Nehl (2004) Factors in *Thielaviopsis basicola* interactions with plants. Proceedings of the 3rd Ausralasian soilborne Disease Symposium, The Barossa Valley, South Australia, pg. 28.

15. Baker, J., S. Al-Jaaidi, D. Nehl, D. Backhouse, M. Katz and L. Pereg Gerk (2004) Transformation of *Thielaviopsis basicola*: A tool to understand the host-pathogen interactions at a molecular level. Proceedings of the 3rd Ausralasian soilborne Disease Symposium, The Barossa Valley, South Australia, pg. 133.

Honours and PhD theses

1. Mijajlovic G. (2004) *Thielaviopsis basicola*: Plant interactions leading to black root rot. Honours Thesis submitted to UNE in January 2005.

2. Al-Jaaidi S. (2007) Transformation of *Thielaviopsis basicola* to study host-pathogen interactions. PhD Thesis submitted to UNE in April 2007.

B. Have you developed any online resources and what is the website address?

No online resource has been developed in this project.

Part 4 – Final Report Executive Summary

Provide a one page Summary of your research that is not commercial in confidence, and that can be published on the World Wide Web. Explain the main outcomes of the research and provide contact details for more information. It is important that the Executive Summary highlights concisely the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

Black root rot, caused by *Thielaviopsis basicola*, is a significant disease threat to cotton, especially in cooler areas and seasons. In just over a decade it has come to affect more than half of the cotton farms in southern Queensland and New South Wales and it is currently found to be present in every surveyed farm. While management strategies based on cultural practices can reduce the severity of the disease and of crop losses, yield can still drop by up to 40% annually and further loss can occur due to increased susceptibility of black root rot-infected plants to other diseases. Thus, there is considerable scope for new disease control methods based on an improved knowledge of the biology of the pathogen and its interactions with cotton. The black root rot fungus occurs as strains that are specific to particular host plants, and must establish a special relationship with living cells of the host root before root rotting can occur. This suggests that there are highly specific biochemical and genetic interactions between the fungus and cotton that are involved in the infection progress. The longer term aim of our multidisciplinary group of researchers is to identify key factors in the molecular interactions between *T. basicola* and cotton roots, to determine whether such interactions could be exploited in disease management. Identification of host-specific interactions during infection could be used to find components of resistance that will increase the efficiency of breeding varieties with enhanced resistance.

In the seed project completed in June 2007, our group developed methods for investigating the interactions between the fungal pathogen and its cotton host, as well as tools for genetic manipulation of the pathogen and for proteome analyses of both the pathogen and cotton roots. The main outcomes in technique developments were (1) the establishment of a genetic transformation protocol for the production of a large number of fungal mutants and the establishment of a procedure to select those mutants affected only in their pathogenicity towards cotton, (2) the establishment of extraction protocols for the purification of both *T. basicola* and cotton root proteins, (3) the development of a method to produce two dimensional protein electrophoresis maps for both *T. basicola* and cotton roots, which resulted in successful production of reference protein maps for both the fungus and cotton roots and (4) the development and optimisation of reliable systems for the study of *T. basicola* interactions with different host plants, which allowed the comparison of the interactions of cotton with pathogenic versus non-pathogenic strains of *T. basicola*.

The techniques developed in the seed project will be adopted in current and future research with the aim of identifying factors responsible for changes in pathogenicity, especially those involved in host-specific interactions. In addition, the protein maps (total cell proteins, also called proteome) will be used in order to identify proteins (and thus, genes) involved in the infection process and to find if the disease could be blocked by reducing plant stimulants that enhance the pathogen, or by inducing plant resistance to the disease. The long term objectives of this project were to find out whether there are host-specific triggers to certain stages of the pathogen infection and whether it is feasible to exploit key steps in the infection process for the development of resistance or other control measures, such as soil amendments.

The development of tools in this seed project allow research into the *T. basicola*-cotton interactions, which could lead to discoveries towards ways of controlling the disease and thus increasing cotton yields, by either breeding cotton towards disease resistance or by producing more effective soil amendments against *T. basicola*.