Cotton Catchment Communities CRC

FINAL REPORT

(due on completion of project)

Part 1 - Summary Details

Cotton CRC Project Number: **1.01.55**

Project Title: Linking cotton-pathogen molecular interactions and black root rot management

Project Commencement Date: 01/07/2007 **Project Completion Date:** 31/12/2011

Cotton CRC Program: The Farm

Part 2 – Contact Details

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Part 3 – IP and In-kind

Since the November 2011 6 monthly report, please outline the additional IP and in-kind that has been generated in the project.

1. Intellectual Property developed within the project.
(What Know-How (New Ideas), Confidential Information, Copyright, Patents or Provisional Patents, Registered Design, Trade Secrets or Trademarks have come from this project to date)

During the life of this project:

- Protein isolation and purification from cotton roots optimised
- Protein isolation and purification from *T. basicola* optimised
- Genetic differentiation among *T. basicola* isolates established
- Highly reproducible pathogenicity test in soil (in pots) optimised
- Methodologies designed for handling and studying *T. basicola* properties
- Genetic transformation of *T. basicola* optimised
- Genetic analysis of mutants optimised
- Knowledge generated on the origin of *T. basicola* isolates and on their interactions with cotton
- Knowledge generated on the response of cotton to *T. basicola* infection

All of the above should be considered as Copyright material until publication and, unless already published in scientific journals, may be shared with other researchers given a research agreement has been made.

2. Project In-kind
(Grower Consultant Ginner or Grower Group In-Kind: Are you conducting part of your project on a cotton farm or in conjunction with an in-kind contribution from a consultant, ginner or Grower Group? Please supply group name - Number of persons involved per week and the number of hours per week involved.)

None since Nov 2011.

Part 4 – Final Report Guide (due at end date of project or 31st May 2012)

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

Background

1. Outline the background to the project.

Black root rot is a fungal disease affecting cotton seedlings upon germination. The fungal soil-borne pathogen, *Thielaviopsis basicola*, is found in almost every cotton farm in Australia and, while it does not kill the plant, it causes substantial loss of yield. It has been considered a significant threat to cotton and other crops in Australia, especially in cooler areas and seasons. The symptomatic stunting of seedlings stays with the plant through to maturity, causing up to 40% yield loss, depending on the season, environmental conditions and cotton growing practices.

The pathogen, *T. basicola*, produces thick walled spores that can survive in the soil for years. It was first detected in NSW in the 1980s and since then quickly spread to all cotton growing areas of NSW. 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), reaching 95% of the fields regularly surveyed in northern NSW in 2004. The 2010/2011 cotton pathology survey, published in the Cotton Pest Management Guide 2011/12, reported black root rot was found in 93% farms visited and in 83% of the fields surveyed in NSW. One of the reasons that makes black root rot hard to control is the wide host range of the pathogen strains, including all cotton varieties as well as many legumes and even some grasses, making most crop rotation option ineffective in reducing fungal spore (reproductive bodies) load in the soil. There are limited management options for reducing black root rot, highlighting the importance of research into this disease.

Until 2004 cotton black root rot research concentrated mainly on describing the infection process and on cultural practices, such as the "come clean, go clean" policy. Nevertheless, periodical inspections of cotton fields show that black root rot has spread to all cotton fields surveyed in NSW. Due to its long surviving, persistent, spores, it is extremely difficult to eliminate the pathogen once it is established in a cotton field. Current management strategies are insufficient for disease control and breeding resistance to *T. basicola* is yet to be established. A comprehensive literature review by L. Pereg (unpublished) on black root rot in plants such as cotton, pansy, red clover, cucumbers, carrots, strawberries and tobacco, revealed that the infection cycle by the pathogen has been well documented in tobacco, pansy and to some extent in cotton using microscopy; the symptoms of the disease on different plants described; the optimal conditions for an outbreak of the disease determined for some crops; limited information exists on the biology of the pathogen and some phylogenetic and taxonomic studies conducted on the pathogen. Thus, while the information required to survey and identify the disease exists, a thorough understanding of the biology of the pathogen and its interaction with the host is required in order to develop ways to effectively control the disease.

Although *T. basicola* as a species has a broad host range, individual strains are only capable of infecting a relatively limited number of host species. This indicates a widespread occurrence of resistance mechanisms (tolerance) in plants, including cotton, that are effective against some 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 technology. To be able to develop and implement innovative measures to manage black root rot more effectively, it is essential to gain more knowledge on the factors controlling the progression of the disease cycle, from its initiation (spore germination) and through to its completion (root rotting and the production of new spores). One of the most important factors in any plant-pathogen interactions is their continuous communication via the exchange of signal molecules. Such signals alter gene expression (function) and protein production in both organisms and consequently allow the infection of the plant by the pathogen; similar communication mechanisms may also be the key to resistance by non-host plants.

Since 2004, research at UNE, led by L. Pereg (in collaboration with D. Backhouse and M. Katz), has been concentrating on developing tools for understanding the biology and ecology of *T. basicola* and studying the interactions of *T. basicola* with its host leading to black root rot. Initially, we proposed to employ a molecular approach to study what controls the progress of the cotton infection process by *T. basicola*. Proteomics and molecular genetics have been used in other systems to study microbial interactions with higher organisms, such as animals and plants. However, initially no molecular genetics or proteomics tool was available for *T. basicola*, nor any protein map from cotton roots. During 2004 to 2007 (CCC-CRC/CRDC seed project 1.01.21) the group (including the postdoctoral fellow J. Coumans and students) concentrated on developing general and molecular tools to be used in studying the *T. basicola*-cotton interactions at the molecular level (genetics and proteomics) as well as in systems that allow large-scale experiments.

Techniques were developed to 1) study the host range of different isolates of *T. basicola* originated from different hosts and geographical regions, 2) genetically manipulate *T. basicola*, e.g. generate random mutants of *T. basicola*, 3) select for pathogenicity mutants and analyse their interactions with their host, 4) extract proteins from the fungus and from cotton roots and study protein differential expression under various conditions, and 5) analyse biological factors that may interfere with the interactions of *T. basicola* with its host. The techniques developed allowed the group to progress to the next step to study the association between this fungal pathogen and its host leading to cotton black root rot, with the intention of generating knowledge that could be used for interrupting the disease cycle.

This project, 1.01.55, has concentrated on further optimising and utilising the newly developed tools to analyse the response of the cotton plant to the presence of the pathogen and the response of the pathogen to the presence of its host. We have also analysed a variety of *T. basicola* strains, differing in their virulence towards cotton, to enhance our understanding of the molecular bases of host specificity and its origin. In this and related postgraduate projects, we have also analysed traits involved in pathogenicity of *T. basicola* towards cotton. Other than knowledge generation and technical developments, this project also contributed to enhancing the human and research capacity of the cotton industry, by involving postdoctoral fellows as well as training PhD and Honours students.

Objectives

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

Overall, long-term objectives are to find out what triggers germination and virulence in the plant pathogen *T. basicola* and then find out how to control it in a way that will contribute to sustainable management of this disease. Identification of factors involved in communication pathways between *T. basicola* and cotton will allow conventional breeding of cotton or the development of genetically modified cotton with reduced susceptibility to black root rot: either by reducing the synthesis of pathogen stimulants or by induced resistance to the pathogen. During communications with plant breeders at the CSIRO Plant Industry, Canberra, L. Pereg have promoted the strategy (contributed information and *T. basicola* strains) of selecting for plant resistance to a *T. basicola* strain-mixture rather than to an individual strain. Such approach would reduce the risk of breeding a plant towards resistance to a pathogenic strain and at the same time increase its susceptibility to another strain of *T. basicola*. Other approaches to affect the interactions between the pathogen and the host may include the use of suitable soil additives, biological or chemical.

More specifically, in this project (1.01.55) we aimed to achieve the following objectives: (1) the analysis of mutants of *T. basicola* with reduced pathogenicity towards cotton, produced in project 1.01.21, for the identification of fungal pathogenicity proteins. This will allow future development of control strategies based on suppressing fungal pathogenicity factors. (2) Identification of plant proteins involved in cotton's response to virulent *T. basicola*. This will allow the discovery of plant factors required for the interaction with the pathogen. (3) Make progress towards understanding the response of the plant to current, successful, treatments and induced resistance in the plant.

We will discuss the results/outcomes of this project with respect to the three objectives listed in the project application:

1. Identification of fungal pathogenicity traits and proteins
2. Identification of plant proteins involved in cotton response to virulent *T. basicola*
3. Progress towards understanding the response of the plant to current, successful, treatments and induced resistance in the plant

Work towards achieving the first objective, "Identification of fungal pathogenicity traits and proteins", has been initially fully conducted as part of this project. Proteins involved in the pathogenicity of virulent *T. basicola* towards cotton were discovered as well as physical traits involved in *T. basicola* pathogenicity towards plants. Most of this work has been published in a highly ranked international scientific journal (see below).

The PhD student G.M Ali took over the genetic analysis of the pathogenicity mutants and will be reporting on this part of the project in his final report (March 2012) but we will include a short extract on his progress too, to give the reader the full picture.

The second objective has been achieved. We have successfully used the methods developed in project 1.01.21 and further optimised it here in order to identify plant proteins involved in cotton response to virulent *T. basicola.* Note that this was a pilot study and that future research using a wider range of conditions could further elucidate a larger number of proteins involved in the plant reaction to the pathogen. Nevertheless, our work generated knowledge and was published in a highly ranked international scientific journal (see below).

There has been substantial progress made toward achieving the third objective, "Progress towards understanding the response of the plant to current, successful, treatments and induced resistance in the plant". Further data analysis may allow publication of the results obtained.

Methods

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

Molecular tools used in this project have been developed in the seed project 1.01.21. Techniques developed in the seed project that were optimised and used in this project include establishments in the fields of proteomics, genomics and phenomics.

Proteomics

Proteomics tools have been developed in the seed project 1.01.21 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 was to use protein mapping to investigate the complex interactions 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.

Proteins are the key elements in the structure and function of all living things. Specific proteins are produced or modified in every organism, including plants and fungi, in response to absolutely every situation the organism faces. Associations between plants and pathogenic microbes will initiate dynamic changes in the protein content of both organisms involved: plants will sense the pathogens and attempt to defence themselves while the pathogens, sensing the plant, will grow towards it, invade it and try to avoid/survive the plant defence

mechanisms. To do all that, both plants and pathogens would have to produce a range of proteins specific for these purposes. These are referred to in the literature as **fungal pathogenicity proteins** and **plant defence proteins**. Other unique proteins may be involved in establishing the association between the pathogen and its host. Researchers attempt to identify such proteins to understand the mechanisms involved in host-pathogen interactions, as these would naturally be key targets in developing disease control measures.

Proteomics techniques optimised and used in this project include proteome (total proteins of an organism) analysis of both *T. basicola* and cotton roots. Protein analysis procedures were used for the identification of fungal proteins involved in the disease cycle and in the plant's response to the pathogen.

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. **Cotton proteins** that are differentially expressed (increase, decrease or modified) in response to virulent *T. basicola* strains are likely to be involved in communication with the pathogen and may be required for completion of the susceptible-host invasion by the pathogen. Therefore, we analysed the cotton root protein maps produced in response to exposure to pathogenic *T. basicola*. Proteins involved in cotton response to Acibenzolar-S-Methyl, a substance inducing disease resistance in cotton, have also been analysed to identify proteins involved in activating mechanisms of disease resistance in cotton.

In order to identify **fungal proteins** required for the pathogenicity of *T. basicola* towards specific hosts we performed (1) Proteome mapping of a collection of *T. basicola* strains with varying degrees of virulence towards cotton (a collection produced in the seed project) as well as (2) analysis of the response of cotton *T. basicola* pathogen to the presence of hosts and non-hosts plants (pathogenicity towards different hosts was established in the seed project). Finding such proteins will pave the way towards developing products that target key mechanisms of fungal pathogenicity, control their expression and thus control the severity of the disease.

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.

The following methods have been optimised:

1. Total protein extraction from cotton roots
2. Total protein extraction from *T. basicola*
3. Protein mapping by two dimensional (2-D) gel electrophoresis
4. Protein purification, analysis by LCMS and identification by database searches

The proteomics tools and research strategies developed in this project can be of benefit in a variety of ways in this and other research projects.

The fungal proteomics tools can be used to/in:

- Identify fungal proteins with different functional roles (e.g. pathogenicity proteins)
- Study the fungal response to different stimulants (e.g. host vs non-host plants)
- Study the origin of strain variation
- Study the fugal response to different treatments (e.g. antifungal substances)
- Developing and optimising such protocols for other fungal pathogens

The plant proteomics tools can be used to/in:

- Identify plant proteins with different functional roles (e.g. plant proteins expressed during its interaction with a pathogen)
- Study the plant response to different stimulants (e.g. pathogenic vs non-pathogenic fungal strains)
- Study the plant response to different treatments (e.g. antifungal substances, induced resistance, nutrient supplements)
- Study the plant response to different environmental conditions (e.g. water restrictions, soil parameters, temperatures)

Genomics

Each protein present in an organism is encoded by a specific gene (or a specific group of genes), which is unique for this protein. Pathogenicity genes encode proteins that are involved in the virulence of a fungal pathogen towards its hosts. The presence and function (expression) of such genes is absolutely essential for the progress of the infection cycle and for inflicting disease by the pathogen. Identifying pathogenicity genes and finding ways to control their function would pave the way to developing pathogen control measures and, thus, disease control measures.

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

1. development of a reliable genetic transformation system for the fungus (achieved in seed project 1.01.21)
2. development of mass-tests to screen for pathogenicity mutants (achieved in seed project 1.01.21)
3. development of verification techniques to confirm reduced pathogenicity in soil (achieved in seed project 1.01.21)
4. analysis and characterisation of pathogenicity mutants (and genes) – undertaken in this project (1.01.55).

Protocols for the genetic manipulation of the fungal pathogen *T. basicola* using a technique called PEG-mediated transformation were developed in the seed project 1.01.21 and five pathogenicity mutants were produced and partially analysed.

Three of the five pathogenicity mutants produced in the seed project were analysed in this project (and in G.M. Ali's PhD project) to identify the pathogenicity gene affected in each mutant (genetical analysis) and the physical and biochemical traits associated with the mutations (phenotypical properties) and thus involved in the pathogenic cycle.

In this project we have attempted to optimise another, less tedious, protocol for fungal mutagenesis (ATMT) however this technique could not be used with *T. basicola*. Therefore, we have successfully concentrated our efforts on further optimising the PEG-mediated transformation technique for *T. basicola*.

The following methods have been tested/used/optimised in this project:

1. *T. basicola* mutagenesis by ATMT (*Agrobacterium tumefaciens* - mediated transformation)
2. *T. basicola* random mutagenesis by PEG-mediated transformation
3. Selection of pathogenicity mutants of *T. basicola*
4. Genetic analysis of mutants by established molecular techniques (Plasmid sequencing, PCR, Southern blotting, plasmid rescue, inverse PCR and Tail PCR) and developing a strategy to determine the most suitable method for analysing the gene affected in each mutant. The original plasmid used in the genetic transformation had to be sequenced for this purpose.

We have also used confirmation techniques, namely ITS analysis (Internal Transcribed Spacers analysis), to ensure that the mutants originated from the parent strain. This method is based on the sequence comparison of highly conserved regions in the genome of different organisms. It was also used to establish the phylogeny (relatedness) of different *T. basicola* strains from different origins and with different host range to complement proteomics data.

The genomics tools developed in this project can be of benefit in a variety of ways in this and other research projects.

Genetic transformation could be used for:

- Random mutagenesis to study different traits when the genes involved are unknown
- Insertion of genetic markers and any other genes of interests into the fungal genome, e.g. providing a convenient tool to observe the interaction of the pathogen with the plant.
- Site directed mutagenesis to knock-out specific genes of interest and study their roles
- Insertion of reporter genes to study gene expression (function) and how it could be controlled

Phenomics

Phenomics is a relatively new term describing the entire physical and biochemical traits of an organism. To elucidate fungal properties related to pathogenicity, several morphological and biochemical tests were conducted, comparing the wild-type *T. basicola* strain to its pathogenicity mutants. To do that we had to develop/optimise the following techniques, some can be adopted for studying other fungal pathogens:

1. Analysis of morphological traits of *T. basicola* pathogenicity mutants
2. Analysis of biochemical traits of *T. basicola* mutants. Properties that are known from the literature to be related to pathogenicity were tested, such as protease and cellulase production.
3. Microscopy methods to follow the interactions of pathogenicity mutants with cotton and determine the critical steps affected in the disease cycle, reducing mutant pathogenicity.

Results

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

We will discuss the results of this project with respect to the three objectives listed in the project application:

1. Identification of fungal pathogenicity traits and proteins
2. Identification of plant proteins involved in cotton response to virulent *T. basicola*
3. Progress towards understanding the response of the plant to current, successful, treatments and induced resistance in the plant

4.1. Objective 1 - Identification of fungal pathogenicity traits and proteins

4.1.1. Genomics

This part of the work is directly linked to previous project (CRDC/CCC-CRC project 1.01.21) in which pathogenicity-related traits and fungal genes have been studied. The group members have been using *T. basicola* wild type strain BRIP40192, recovered from diseased cotton host in the cotton-growing region of Narrabri and all pathogenicity tests were done using cotton variety Sicot189, which is commonly affected by black root rot.

Five *T. basicola* mutants (P849, P16, P954, P737 and P888), that were affected in their pathogenicity towards cotton and showed very mild black root rot symptoms on seedling-roots, have been used in this project for further analysis of the pathogenicity genes affected.

4.1.1.1. Optimisation of gene analysis technique

Five pathogenicity mutants, originally produced in a previous project (CRDC/CCC-CRC project 1.01.21) were used for optimisation of the genetic analysis the mutants. All of the five mutants were produced by random insertion of a plasmid into the fungal genome (*T. basicola* BRIP40192, originally isolated from a cotton host). Since the location and mode of insertion of the plasmid differs in each of the mutants, different techniques had to be developed to analyse the affected genes in each case.

Preliminary analysis revealed the probable location and mode of insertion of the plasmid into the fungal genome in each of the mutants. This was done using two techniques:

1) Southern blot analysis, which detects whether there was one or more insertions of the plasmid into the fungal genome and also whether the plasmid is inserted in the same or different locations in the fungal genomes of the different mutants.
The information obtained from this analysis allowed us to select for three mutants out of five (P16, P954 and P849) for further genetic analysis, as only these mutants appeared to contain a single plasmid insertion in their genomes (this is essential to allow any further genetic analysis).

2) PCR amplification technique, which can inform us whether the integration of the plasmid affected essential plasmid-borne motifs.
This is important information to assist us in selecting the correct strategy to analyse the affected gene. If essential plasmid motifs (such as the origin of replication or the antibiotic-resistance gene) were affected, plasmid rescue technique would not be suitable and alternative methods should be sought.

Based on the results from the above-mentioned analyses, we applied and optimised most suitable techniques for analysing the gene affected in each mutant. This work has continued by the PhD candidate G.M. Ali.

The plasmid-rescue technique has been applied in order to analyse the *T. basicola* pathogenicity-mutant P16, and the inverse-PCR technique in order to analyse the *T. basicola* pathogenicity-mutants P954 and P849. In both methods the mutant's genomic DNA is digested (cut) by restriction enzymes. A careful selection of the restriction enzymes is required for optimal results. Following the digestion of the genomic DNA, the resultant linear fragments are re-ligated, each forming a circular DNA.

Where the application of the plasmid-rescue technique is possible, such as with P16, the circular DNA is then transformed into the bacterium *E. coli* as a way of amplifying its quantity. As the original plasmid we used for transformation contains an antibiotic-resistance gene, we can select for the circular DNA containing the plasmid by growing the *E. coli* on antibiotic-containing medium. By careful selection of the restriction enzyme, used to digest the genomic DNA of the mutant, we can ensure that some fungal DNA from the mutated fungal gene will be rescued together with the plasmid. We can then sequence the region of the rescued plasmid containing the fungal DNA sequences from the mutated gene.

Where the application of the plasmid-rescue technique was not possible, such as with the mutants P954 and P849, we employed the Inverse-PCR and/or tail-PCR techniques. When using these methods we did not transform *E. coli* with the circular DNA, but used carefully designed PCR primers to amplify regions of the fungal genome directly adjacent to the inserted plasmid in the mutant. We then cloned these amplified regions into commercially available plasmids, which allowed us to maintain the DNA fragments, stored them and subjected them to sequencing. We could then sequence part of the fungal gene/genetic-motif affected in these mutants and try to identify the pathogenicity related gene affected

Using the plasmid-rescue technique, the inverse PCR technique and polymerase chain reaction (PCR) assays, G.M. Ali reported significant progress in the identification of two putative pathogenicity genes from the reduced-pathogenicity mutants of *T. basicola*, P16 and P849. Five hundred and 1800 base pair of genomic DNA sequences were recovered from P849 and P16, respectively and can be used as the basis for further work required for full gene analyses. PCR analytical assays confirmed these DNA sequences were originated from *T. basicola*. Further results from this work will be reported by G.M. Ali.

4.1.1.2. Testing additional transformation methods - ATMT

In this project we have been using the PEG-mediated transformation technique to generate *T. basicola* mutants, in particular pathogenicity mutants. This method has been found useful, however, it is tedious and time consuming and the analysis of the resultant mutants has been proven difficult. Other methods have been published for fungal transformation, with the *Agrobacterium tumefaciens* - Mediated Transformation (ATMT) seeming as the most simple to conduct and less complicated with regard to the genetic analysis that follows.

Three students have attempted to adopt and optimise the ATMT method for *T. basicola*, including the PhD candidate G.M. Ali (who will further report on it in his final report to the CCC-CRC). We have been using a variety of ATMT-plasmids (e.g. pBGgHg, pBHt2), *A. tumefaciens* strains (LBA4404, AGL1) and a variety of methods we have obtained from researchers working on ATMT worldwide, however we have not succeeded with transforming *T. basicola* using this method.

The aim of the ATMT is to randomly insert a known linear fragment of DNA into the fungal genome to create random mutations. The initial steps of the ATMT procedures, used to mutate *T. basicola,* seemed to be working properly as indicated by the growth of some hygromycin (antibiotic) resistant fungal strains (the inserted DNA carries a hygromycin-resistance gene). However, those strains were proved to be unstable, lost their hygromycin-resistance and genetic analyses, using southern-blotting, found no traces of the inserted DNA in their fungal genome.

From consultations with both the literature and other researchers, it seems that some fungal strains cannot be transformed using this method (ATMT). The reason for it is still unclear. We have consulted with a group in Melbourne that regularly performs ATMT and they reported success with some filamentous fungi but not with others, with no transparent reason. We, therefore, decided to continue with the PEG-Mediated transformation technique for *T. basicola* (originally optimised for *T. basicola* in the seed project 1.01.21) and another member of the group (Honours student R. Gentile) further optimised this technique for *T. basicola* and was successful with generating pathogenicity mutants.

4.1.2. Phenomics

Reduced pathogenicity of five *T. basicola* mutants, produced in the seed project 1.01.21 (P16, P849, P954, P888 and P737), towards cotton was confirmed in both soil and agar test systems. The phenotypes of the five mutants were also fully investigated with emphasis put on properties known to be related to pathogenicity in other fungal pathogens and properties important for survival in the soil (e.g. melanin production, salt and pH tolerance).

T. basicola produces two types of spores (Figure 1), which are the resting bodies of the fungus in the soil used for infecting plant seedlings. One type, the clamydospores, produces melanin, a dark pigment, which was implicated in pathogenicity in other fungal pathogens. We tested the mutants for spore morphology and melanin production using light microscopy.

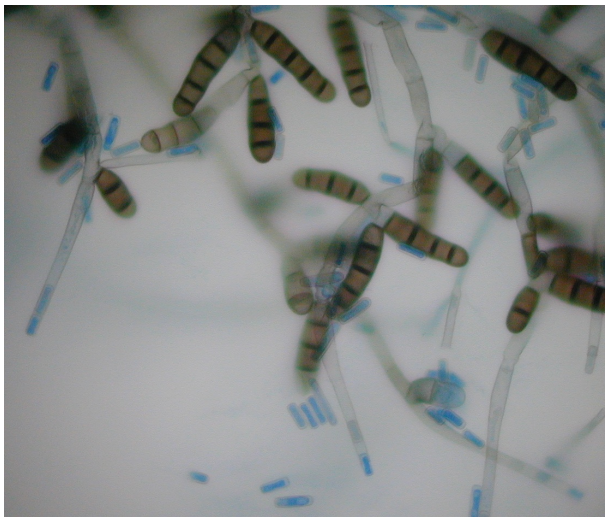

Figure 1. Two types of *T. basicola* spores: dark, melanised chlamydospores organised in chains and individual conidiospores (endoconidia) released from the cylindrical conidiophore.

4.1.2.1. Morphology of chlamydospores and melanin production

The colony morphology of the wild-type *T. basicola* and the five pathogenicity mutants is shown in the figure below. Out of the five mutants that showed reduced pathogenicity, three (P16, P849 and P954) had reduced pigmentation of their chlamydospores on solid medium and did not synthesise a dark brown pigment (melanin) when grown in liquid cultures. P737 and P888 produced chlamydospores that were similar in appearance to those of the wild type, but had a darker brown pigment than the wild type (see Figure 2 below).

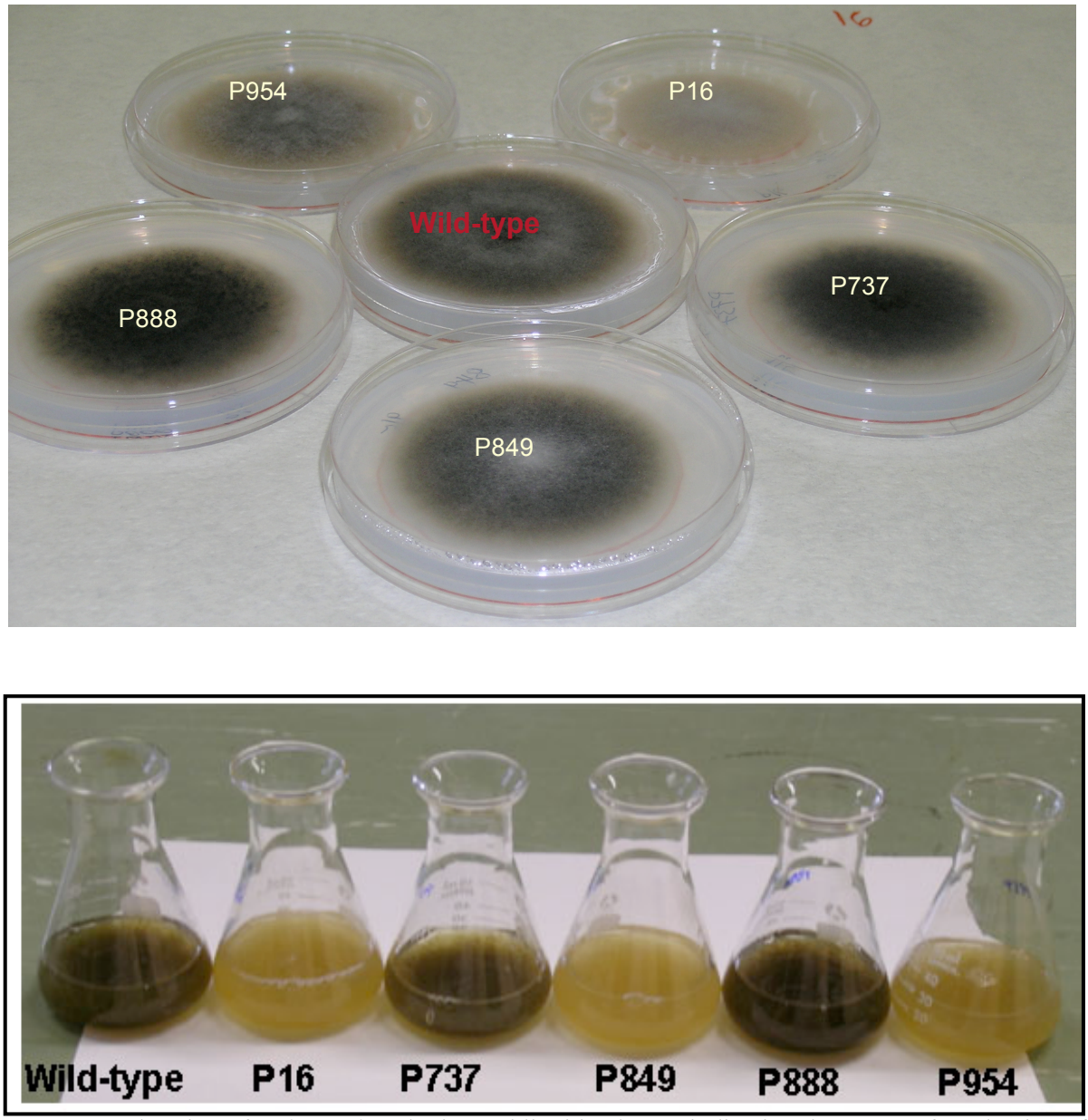

Figure 2. Coloration of *T. basicola* colonies and liquid cultures, indicating the extent of production of the dark pigment melanin.

Further investigations of mycelia (fungal bodies) in liquid cultures, by light microscopy, demonstrated morphological differences between the chlamydospores of the wild-type strain and the mutant 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 (see Figure 3 below - A) that will become a spore. However, in P16, the majority of chlamydospore chains were abnormal (see Figure 3 below-B). They consisted of 5-7 light brown segments with each segment containing an individual light brown semi-cylindrical cell.

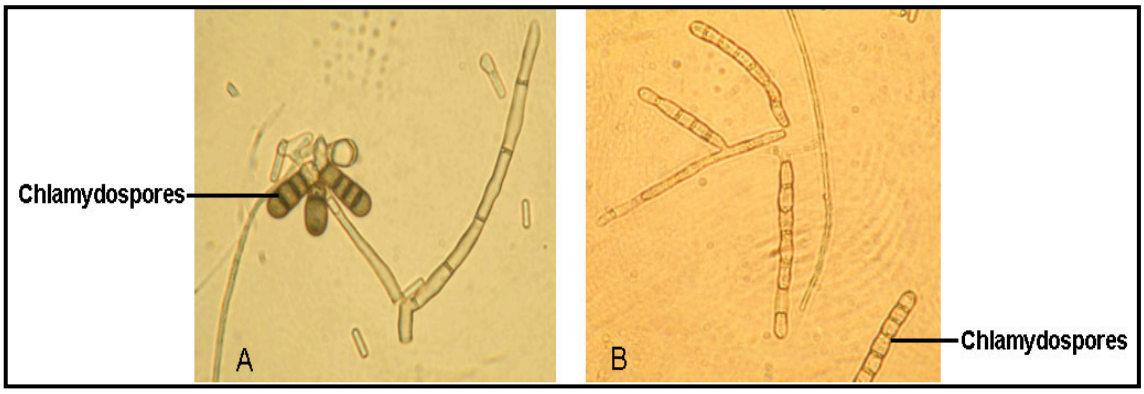

Figure 3. Chlymydospores of wild type *T. basicola* and the mutant P16.

Mutants P849 and P954 chlamydospores also showed 3-4 thick segments but they were light brown. For P849, a large number of chlamydospores were always seen tightly clustered together. Many of these spores appeared abnormal, having only one swollen spore per chain, which showed reduced melanin content. For P954, the overall melanin content of the spores also seemed reduced. The chlamydospores of P888, resembled the wild type ones in morphology, but had higher melanin content. For P737, there was often only one chlamydospore per chain however, they appeared normal in morphology and still showed comparable melanin content to the wild type (Figure 4).

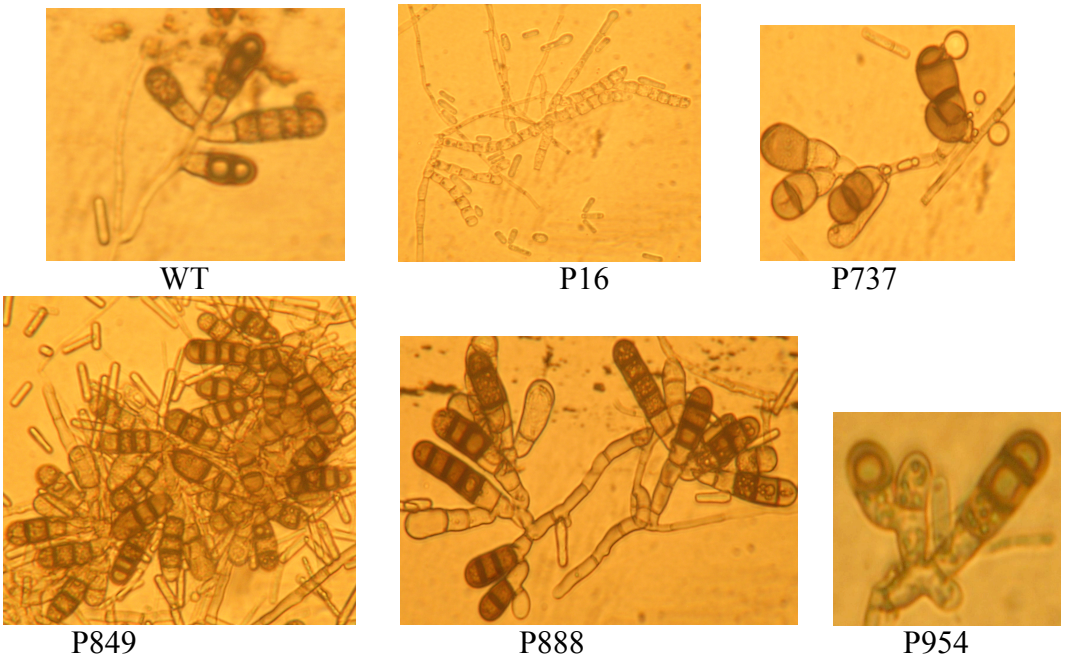

Figure 4. Chlamydospore Morphology. Chlamydospores of the five *T. basicola* mutants and wild type as viewed under the compound microscope (under x400 magnification).

4.1.2.2. Growth rate and spore count

Analysis of all mutants for colony diameter on agar plates showed only slight differences from the wild type (see Figure 5 below) that cannot provide an explanation to their reduced pathogenicity. Thus, the reduced pathogenicity is most likely as a result of mutation in virulence related genes rather than in a general "house-keeping" gene (a gene that may affect growth in general).

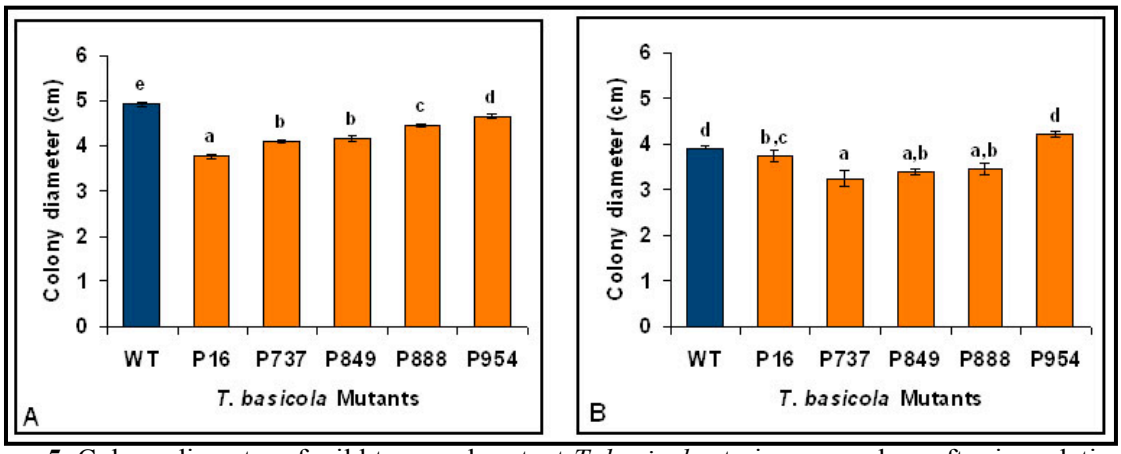

Figure 5. Colony diameter of wild type and mutant *T. basicola* strains seven days after inoculation of growth plates.

Spore production varies greatly among the wild type and the mutants (see Figure 6 below), with no positive correlation between the number of spores produced and the level of pathogenicity. For example, the mutant P849 produced higher number of endoconidia than the wild type, even though it shows reduced virulence.

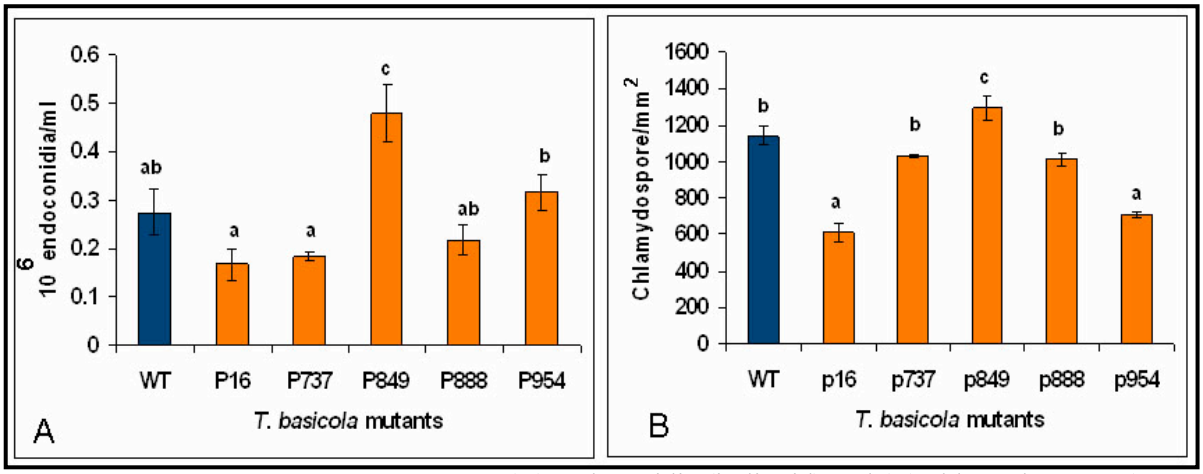

Figure 6. Enumeration of fungal spores: (A) endoconidia (in liquid) and (B) chlamydospores (on solid medium) of wild type and mutant *T. basicola* strains.

4.1.2.3. Tolerance of osmotic pressure and medium pH

Changing the pH of the medium from pH 5 to pH 10 had no effect on the growth rates of wild-type *T. basicola* or its five pathogenicity mutants. Wild-type *T. basicola* showed slightly faster growth compared with the mutants when grown on PDA medium (common rich medium for fungal growth). P16 showed the slowest growth. The growth rate of wild-type *T. basicola* and all mutants decreased as the concentration of NaCl increased (Figure 7), however P16 seems the least affected by increased salinity.

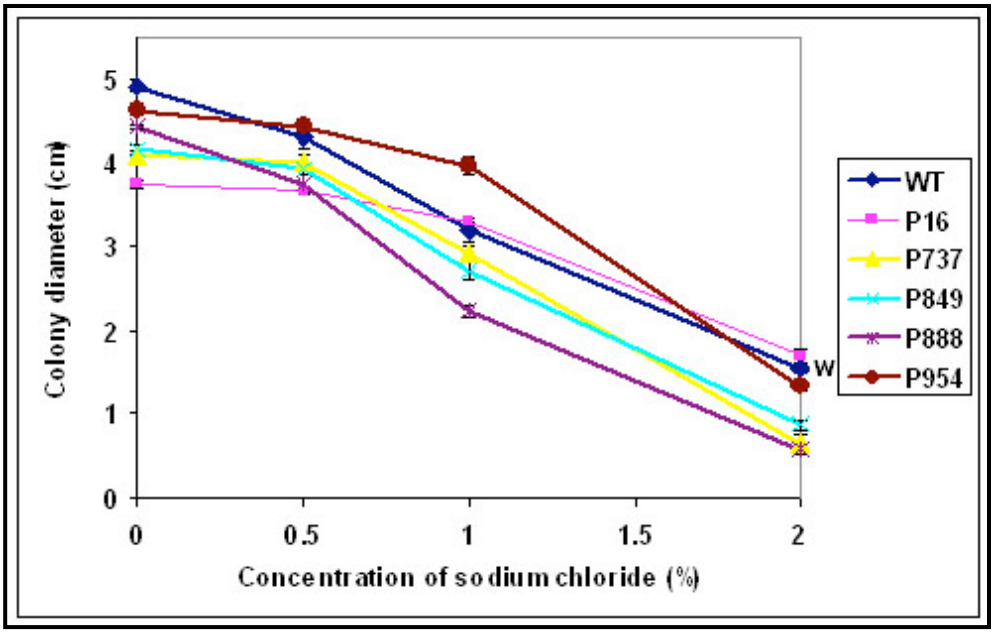

Figure 7. Effect of osmotic pressure on vegetative growth of *T. basicola* pathogenicity mutants. Mutants and wild type were grown on PDA amended with 0.5%, 1%, or 2% NaCl for seven days and the colony diameter was measured. Controls lacked sodium chloride. Values are the means of four replicates. Vertical bars represent standard errors of the mean from three combined replicates.

4.1.2.4. Interactions of the five pathogenicity mutants with cotton

Both wild type and the five pathogenicity mutants of *T. basicola* were analysed for properties known to be associated with virulence in other pathogens. These included the production of (plant) cell-wall degrading enzymes, such as cellulases, pectinases and proteases. The mode of interaction of the mutants with the cotton seedling was compared with that of the wild-type pathogen in order to find out the step in the disease cycle, which is interrupted in each mutant. This work has been undertaken by the PhD candidate G.M. Ali and only the highlights will be summarised in this report.

Production of plant cell-wall degrading enzymes

Cellulases and pectinases are enzymes that were found to be related to fungal invasion of plant tissues – they allow the fungi to digest cellulose and pectin in the plant cell wall, providing a mean of entrance of the fungus into the cells. Utilisation of cellulose or pectin as sole carbon sources by fungi suggest the production of cellulases and pectinases and could indicate the possibility that these enzymes are involved in pathogenicity. G.M. Ali reported that both the wild type and the mutants were growing on pectin, cellulose and glucose as sole carbone source. They did not grow on minimal medium without any carbon source. This means that all are probably producing pectinase and cellulase and these may be involved in patogenicity. It seems that the mutations affecting the virulence of the five mutants are not in their ability to produce these cell-wall degrading enzymes. However, we could not conclude from the results of the above experiment whether plant cell wall degrading enzymes may be involved in the pathogenicity of *T. basicola* towards plants.

Cotton root colonization by *T. basicola* wild type and pathogenicity mutants:

Previous studies indicated that *T. basicola* completed its life cycle on cotton within 72 hour post-inoculation, when endoconidia and chlamydospores appeared on the surface of the inoculated roots (Mauk, PA and Hine, RB. 1988. Infection, colonization of Gossypium hirsutum and G. Barbadense, and development of black root rot caused by *Thielaviopsis basicola*. Phytopathology 78:1662-1667). The report also showed that tissue colonisation was intracellular and occurred immediately after the fungus penetrated the cotton roots. Important information on host-pathogen interactions and fungal pathogenicity traits can be generated by studying fungal mutants that are deficient in the ability to cause disease. The *T. basicola* mutants generated in the CRDC/CCC-CRC project 1.01.21 cause very mild black root rot symptoms compared to the wild type. We compared the interaction of *T. basicola* pathogenicity mutants with cotton root to that of the wild type to ilucidate at which stage in the disease cycle the interaction is affected.

This work was carried out by the PhD candidate G.M. Ali. Seedling inoculation was achieved using the dipping technique, optimised for *T. basicola* pathogenicity tests in the seed project 1.01.21. Evaluation of root colonisation was done after sectioning of the roots. Only mutant P849 was also tested in whole mounts since it induced the mildest black root rot symptoms on cotton compared to the other mutants.

Light microscopy studies established that the *T. basicola* pathogenicity mutant P849 was unable to successfully colonise cotton roots. Three-day-old cotton (cv. Sicot 189) seedlings, which were inoculated with endoconidia from either the wild type *T. basicola* strain BRIP 40192 or its pathogenicity mutant P849, showed disease symptoms with the wild type but less so with the mutant. Both the wild type and the mutant strains showed similar infection process up to 48h after inoculation. A remarkable difference between the wild type and the mutant strains occurred 72h after inoculation when mutant strain P849 failed to colonise the cortical tissue. In roots inoculated with P849, no evidence of *T. basicola* structures was observed past the epidermal and sub-epidermal cells, which stained dark blue with the stain toluidine-blue and appeared to have a denser cytoplasm. None of the mutant strains had colonised the vascular tissue 72h after inoculation. Though the precise nature of the tannin-like substance (which stained blue) in the epidermal cells is unclear, we suspect that this chemical played a role in the resistance reaction of cotton roots to infection by *T. basicola* strain P849.

Although mutant strains P16 and P954 colonised the root cortical tissue, large numbers of invasive hyphae were not evident. One possibility is that the mutant strains did not produce the required quantities of cell-wall degrading enzymes. On the contrary, extensive death of cells in the cortical tissue 72 h after inoculation with the wild type strain suggests activity of cell-wall degrading enzymes.

4.1.3. Proteomics

In the seed project 1.01.21, interesting preliminary observations were made based on two-dimensional gel electrophoresis (2-DE) separation of cotton root proteomes. These results suggested that (1) *T. basicola* is capable of adapting its proteome to germinate and grow according to nutrients available in its environment and (2) new proteins are expressed as early as one day in the cotton root after *T. basicola* infection. Therefore, two of the main objectives in this subsequent project (1.01.55) were to confirm these results and to identify proteins of interest by mass spectrometry.

4.1.3.1. Identification of proteins involved in *T. basicola* virulence towards cotton

This part of the project originally started in collaboration with J. Harvey, UQ and due to misfortunate circumstance was completed by our group.

The results of this section were published in a highly ranked international journal (J.V.F. Coumans, J. Harvey, D. Backhouse, A. Poljak, M.J. Raftery, D. Nehl, M.E. Katz and L. Pereg. Proteomics assessment of host-associated microevolution in the fungus *Thielaviopsis basicola* isolates. *Environmental microbiology*, 13:3 576-588).

The aim of this study was to establish whether comparing the proteomes (total protein mapping) of various *T. basicola* isolates, differing in their host range, could shed light on phenotypic factors related to pathogenic specialisation. Fungal genetic elements and entire proteome analyses were used to determine the relation between *T. basicola* variants and to provide information on the main derives in the evolution of the species.

The full information on the results of this study can be found in the published article attached to this report. A summary of the major highlights is given below.

Twelve isolates of *T. basicola* isolated from different hosts (cotton, lettuce, carrot) and geographical regions were analysed by ITS sequencing, total protein mapping and testing their level of virulence towards cotton.

Analysis of the ITS region of these strain and comparison to *T. basicola* isolates from other groups revealed that isolates can be grouped based on host of origin irrespective of geographical origin (Figure 8). Evidence was found to suggest that cotton, lettuce and carrot strains all belonged to the same species and phenotypic differences between them represent intra-species variations.

At the proteome level a high degree of diversity was apparent among the *T. basicola* isolates and, in agreement with the ITS results, hierarchical clustering analysis of the data also demonstrated a close correlation between the proteome and the host of origin (Figure 9). An additional analysis of the data indicated that only approximately a third of the proteome is common to the 12 *T. basicola* isolates and that this number increased dramatically when isolates from a common host are considered.

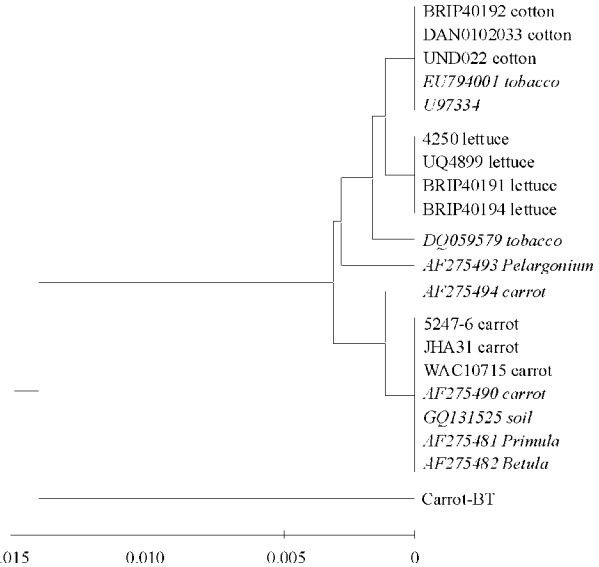

Figure 8. UPGMA distance tree of *T. basicola* isolates based on ITS sequences. Host or substrate of origin is shown where known. Names in italics represent sequences obtained from databases. The tree was rooted with the sequence from an isolate of *T. thielavioides* (Carrot-BT).

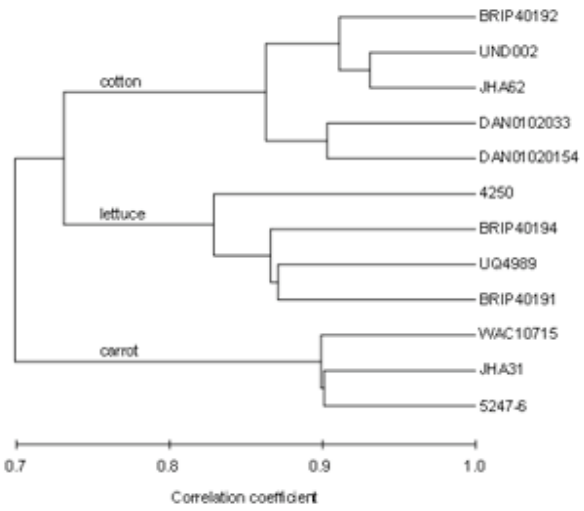

Figure 9. Classification of the proteomes from the twelve *T. basicola* isolates. Hierarchical clustering was performed using the nearest neighbour method with the Pearson correlation as a similarity measure.

In order to gain further insight into the biochemical differences between these isolates, we also identified proteins unique to each isolate and proteins differentially expressed among isolates (Table 1). Moreover, we postulated that the fungal isolates have adapted to maximally benefit from the host plants to assure their survival, an adaptation, which is reflected in their proteomes.

Table 1: Proteome variability between *T. basicola* isolates originated from a same host.

Host	Protein spots unique[a]	Protein spots not detected[b]	Protein spots differentially expressed[c]		Protein spots preferentially expressed[d]	
			Over	Under	Over	Under
Cotton	17	27	15	5	2	1
Carrot	48	58	185	10	37	6
Lettuce	10	27	17	5	4	0

[a] Protein spots found in all isolates originated from a same host but absent in all other isolates.
[b] Protein spots not detected in any isolates from a same host but present in all isolates originated from the two other hosts.
[c] Protein spots significantly ($P < 0.05$) over or under expressed in isolates originated from a same host.
[d] Subset of the protein spots differentially expressed in isolates originated from a same host but with at least a 2-fold change in all the isolates within a host group when compared to the two other host groups.

Based on this hypothesis, we explored the possibility that the expression level of specific protein spots in different isolates would correlate with the level of virulence against cotton. As shown in Figure 10, these isolates possessed different levels of virulence towards cotton. Using a Spearman's rank correlation, we found that 52 protein spots were negatively correlated with the level of virulence while 30 protein spots were positively correlated with the level of virulence.

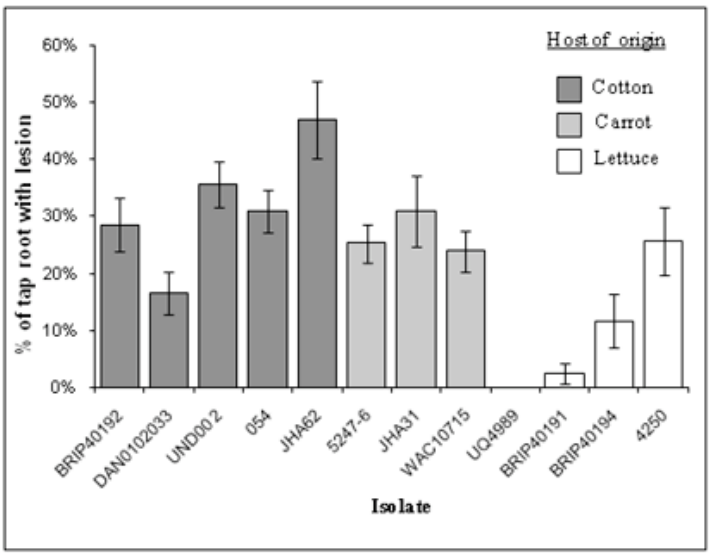

Figure 10. Pathogenicity to cotton of *T. basicola* isolates varied according to the host of origin. Vertical lines denote standard errors of the mean from three combined experiments (n = 15) (J. Harvey's data)

Further purification and analysis of 43 specific proteins have established that the majority of these proteins were involved in primary and secondary metabolism of *T. basicola*. Being a soil inhabitant, *T. basicola* can survive extended periods of time in the absence of host, however the survival of this hemibiotrophic fungus also depends on its close association with its host, therefore some of these identified proteins may be important in plant disease establishment.

Examples of proteins identified, related to host specificity and virulence differences are:
(1) A hypothetical protein with a Rossman-fold NAD(P)(+)-binding protein domain, which represented up to 1.8% of the total protein expression level on gels containing protein extracts from lettuce isolates, but only 0.5% in carrot and less than 0.1% in cotton isolates. Such a large difference in expression level suggests important functional variation between isolates originating from a particular host.
(2) A glyceraldehyde-3-phosphate dehydrogenase which has been reported as a virulence factor in several microbial pathogens and was characterised as an adhesin binding to host extracellular matrix components allowing infection.
(3) An arginase that may reduce Nitric Oxide synthesis, a substance produced by the host as a defence mechanism against pathogens, as previously suggested for the bacterium *H. pylori* and the pathogenic fungus *Histoplasma capsulate*.
(4) A tetrahydroxynaphthalene reductase, an enzyme that operates in the fungal melanin biosynthesis pathway, which may might be responsible for existence of colony pigmentation variation and account for virulence differences between *T. basicola* isolates. Melanin may also contribute to the survival of this pathogen in adverse conditions, as previously suggested for other plant pathogens.

In summary, our genomic and proteomic analysis provides evidence that in Australia, *T. basicola* originated from descendents of single strains or groups of closely related strains associated with specific hosts. MASCOT MS/MS ion searching, *de novo* sequencing and sequence similarity searches identified host-specific differentially expressed proteins and proteins with expression levels that could be correlated with virulence against cotton. Identification of these proteins, listed above, provided insight into the biochemical diversity of *T. basicola*. This work has laid the foundation for further studies to characterise the virulence-related proteomic differences within the *T. basicola* cotton-adapted strains and may help in the design of better strategies for the control of black root rot in cotton.

4.1.3.2. Identification of the *T. basicola* proteins that are specifically expressed in presence of host root

The results of this section were published in a highly ranked international journal (J.V.F. Coumans, P.D.J. Moens, A. Poljak, S. Al-Jaaidi, L. Pereg and M.J. Raftery (2010) Plant extract induced changes in the proteome of the soilborne pathogenic fungus *Thielaviopsis basicola*. Proteomics, 10 (8): 1573-1591).

The full information on the results of this study can be found in the published article attached to this report. A summary of the major highlights is given below.

This paper presents a multiple comparison of the response of *T. basicola* to host versus non-host plant exudates. The aim of the study was to find whether *T. basicola* germination and growth are influenced differentially by the host plants compared to non-host plants and whether specific fungal proteins are stimulated or repressed in reaction to different plants. Proteins uniquely expressed in the presence of a host are highly likely to be involved in virulence. Identifying such pathogenicity proteins (and thus genes) is a step towards controlling their activity and, thus, towards developing ways to control the disease.

We exposed a *T. basicola* isolate, pathogenic towards cotton, to extracts of several plants, including cotton (host), lupin (host), hairy vetch (non-host) and wheat (non-host). We found that *T. basicola* colony diameters (Figure 11) and colony morphology (Figure 12) were significantly different in the presence of the various root extracts and that growth was significantly favoured in the presence of host extracts compared to growth in the presence of non-host extracts.

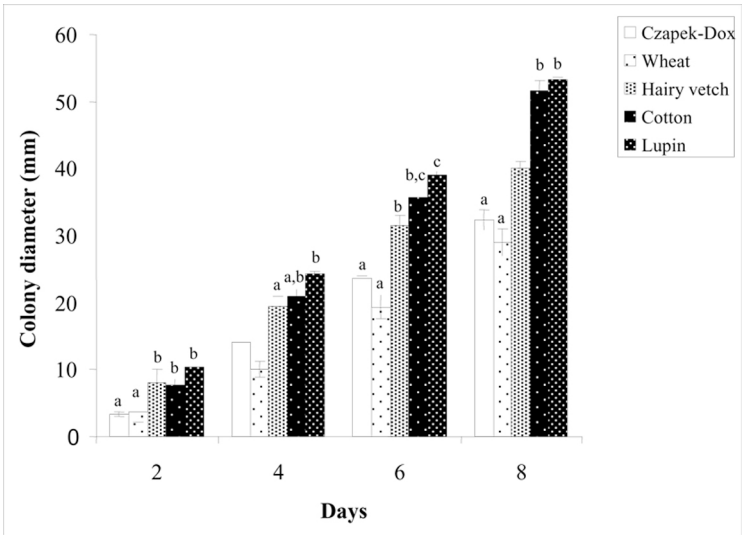

Figure 11. Growth rate assessment of *T. basicola*: *T. basicola* was grown at 25°C on Czapek-Dox agar medium and Czapek-Dox agar medium supplemented with non-host (wheat and hairy vetch) and susceptible host (cotton and lupin) root extract (1:10 v:v dilution) and the colony diameter recorded. Columns labelled with the same letters do not differ significantly (Duncan's test ($P<0.05$)). Bars represent standard errors of the mean from 3 combined replicates.

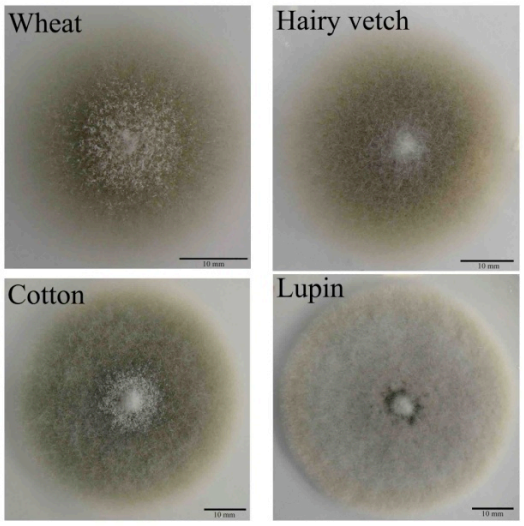

Figure 12. Colony morphology of *T. basicola* in the presence of non-host and susceptible host root extracts. *T. basicola* was grown for 8 days at 25°C on Czapek-Dox agar medium (data not shown) and Czapek-Dox agar medium supplemented with non-host (wheat and hairy vetch) and susceptible host (cotton and lupin) root extract (1:10 v:v dilution).

On the other hand, hierarchical clustering analysis of protein profiles of *T. basicola* indicated that the protein profile was not necessarily dependent on the fungal exposure to host versus non-host extracts. It was more dependent on the species of plant used (Figure 13).

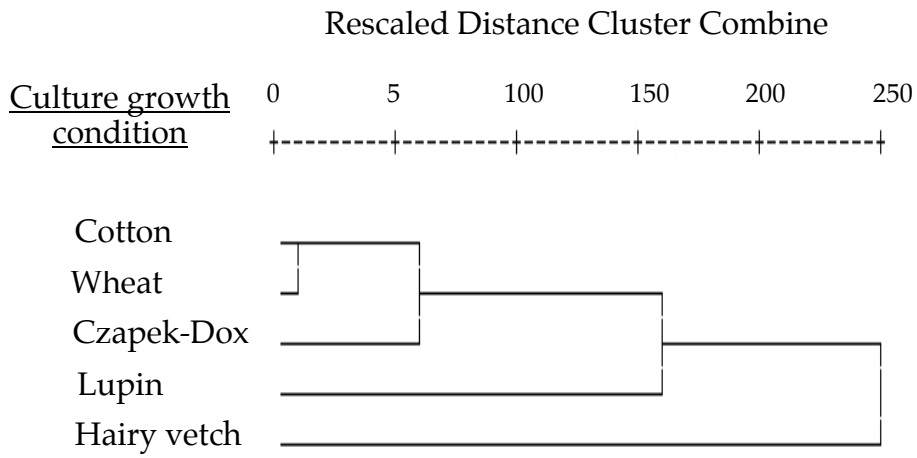

Figure 13. **Classification based on the proteome (total protein profile) of *T. basicola*:** *T. basicola* was cultured in Czapek-Dox control and Czapek-Dox supplemented with wheat, hairy vetch, cotton and lupin root extract. Total protein profiles were compared. Hierarchical clustering was performed using the nearest neighbour method with the Pearson correlation as similarity measure.

The proteomic response of *T. basicola* to the presence of particular root extracts was mapped and compared to its response to other root extracts. Unique protein spots and protein spots with at least a 2-fold difference between root extracts were identified.

The genome of *T. basicola* is not sequenced and to date there is no proteome database available. Protein identification is therefore challenging and depends exclusively on cross-species similarity searching. Using a strategy combining MS/MS ion searches, peptide *de novo* sequencing and BLAST sequence-similarity searching, we could confidently assigned peptide sequences to 41 of the protein spots of interest, the majority of these peptide sequences being of fungal origin. Based on their putative function we found that most of the identified proteins had a role in primary metabolism while the others were implicated in

diverse functions including genetic information processing, cytoskeleton organization, ligand binding and stress response.

In the presence of hairy vetch root extract, four over-expressed proteins were identified as hypothetical proteins highly similar to the pyridoxine biosynthesis protein PDX1 and three additional putative proteins containing a pyridoxal phosphate binding site. The protein PDX1 is highly conserved between different organisms, and is a key enzyme in vitamin B_6 *de novo* biosynthesis. PDX1 jointly with PDX2 function as aminotransferases in which PDX2 produces ammonia from glutamine and PDX1 combines ammonia with intermediates of glycolysis and the pentose phosphate pathway to form pyridoxal 5-phosphate (PLP), the biologically active form of vitamin B6. It is worth pointing out that three other proteins over-expressed contained peptides belonging to proteins involved in the pentose phosphate pathway. Why increased of vitamin B_6 *de novo* biosynthesis occurs in the presence of hairy vetch root extract is unclear. It is important to point out that PLP is an essential metabolite in all organisms and is required for more than 100 enzymatic reactions and that lack of this vitamin is therefore lethal.

In the presence of lupin root extract five *T. basicola* proteins associated with pyruvate metabolism were over-expressed. Three of these protein spots contained peptides assigned to hypothetical proteins containing a conserved domain belonging to malic enzyme. Its function in pyruvate metabolism is well known as well as its vital role in *de novo* lipid biosynthesis through the supply of NADPH. We have used fluorescence microscopy to confirm the presence of lipid bodies in the fungal hyphae and a quantitative analysis confirmed an increase in their prevalence in *T. basicola* exposed to lupin root extract (Figure 14 A, B, C).

To test whether the root extract content in carbon, nitrogen and lipids could account for this increase in lipid body formation, their relative concentrations in the root extracts were determined. We found that lupin and hairy vetch root extracts contained larger quantities of nitrogen and carbon compared to cotton and wheat root extracts. A rapid estimation of the lipid content in the different root extracts by Nile red fluorescence intensity measurements revealed that hairy vetch and lupin contained larger quantities of lipids (Figure 14 D) compared to wheat and cotton and that root extracts from cotton contained more polar than neutral lipids (Figure 14 E).

From these results, it can be concluded that the growth and lipid content differences observed in *T. basicola* are unlikely due to a direct nutritional effect since while lupin and hairy vetch root extracts contain similar amount of carbon, nitrogen and lipids, only lupin induced higher lipid body formation in *T. basicola*. Moreover, we suggested that *T. basicola* host plants may contain compounds, such as flavonoids and strigolactones, which have been reported to influence spore germination, hyphal growth and differentiation in several symbiotic and pathogenic plant-fungus interactions, that may act as plant signals to stimulate *T. basicola* spore germination and growth possibly even before infection.

Several protein spots contained peptide sequences assigned to proteins involved in cytoskeleton organization (cytoskeleton is important in cellular morphology). These correspond to protein spots differentially expressed in the presence of hairy vetch and lupin root extract. To assess if the these proteins may contribute to differences in hyphal morphology, we carried out a microscopic study of *T. basicola* endoconidia cultured in the presence of the different plant root extracts (Figure 15).

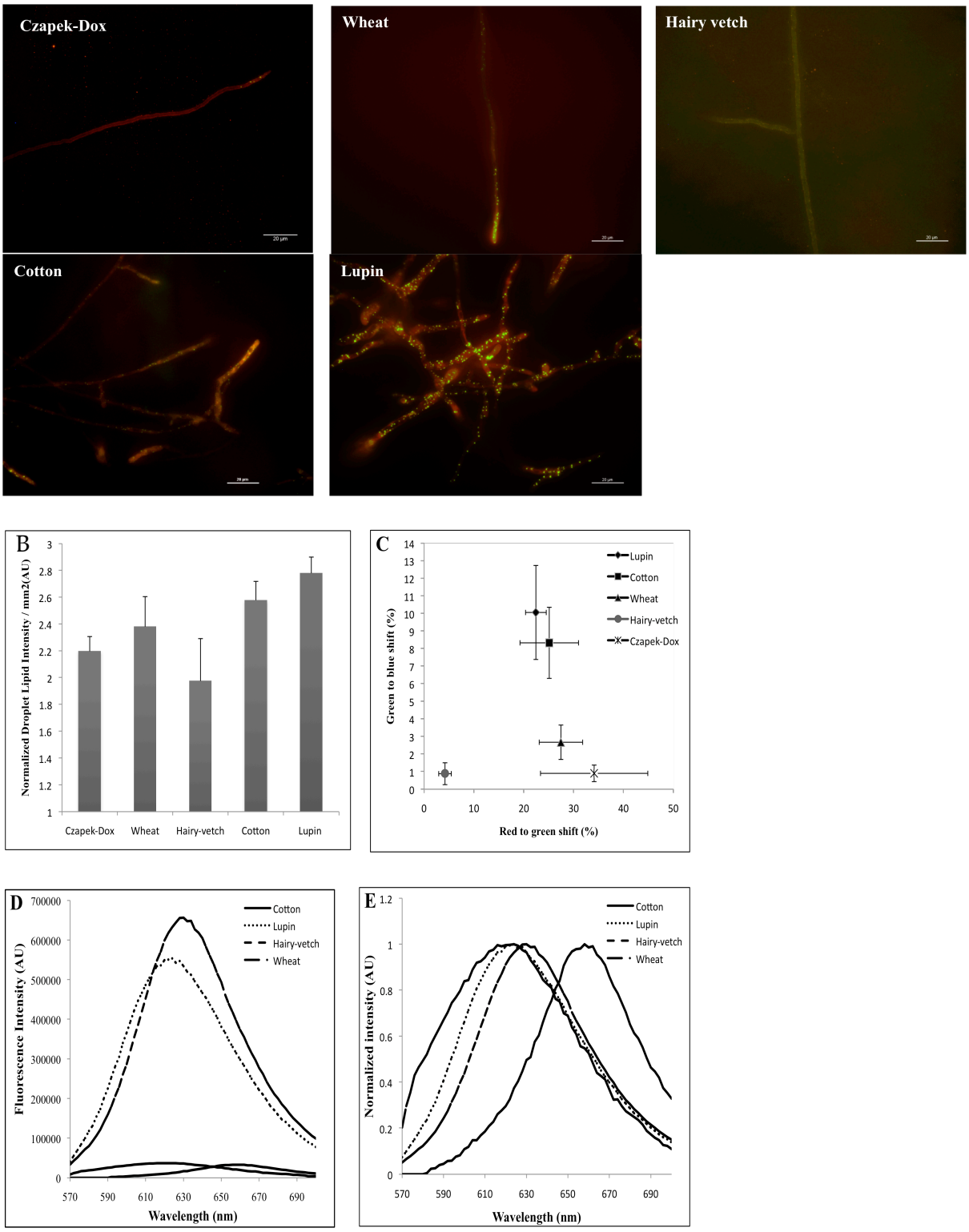

Figure 14. Microscopic photographs of Nile red fluorescence of *T. basicola*. (A) *T. basicola* was grown for 24 h at 25°C in Czapek-Dox and Czapek-Dox supplemented with non-host (wheat, hairy vetch) or susceptible host (cotton, lupin) root extract (1:10 v:v dilution) and stained with Nile red (0.4 ug/ml). (**B**) Analysis of lipid content of lipid bodies (data are mean ± sem) relative to the membrane and diffuse cytoplasmic staining of the cells. (**C**) Analysis of the colour shift of the lipids present in the lipid bodies normalized to the membrane and diffuse cytoplasmic staining of the cells (data are mean ± sem). (**D**) Emission spectrum of Nile red incubated with plant root extracts after background subtraction. (E) Normalized spectrum showing the wavelength shift in the maximum intensity peak for the different root extracts.

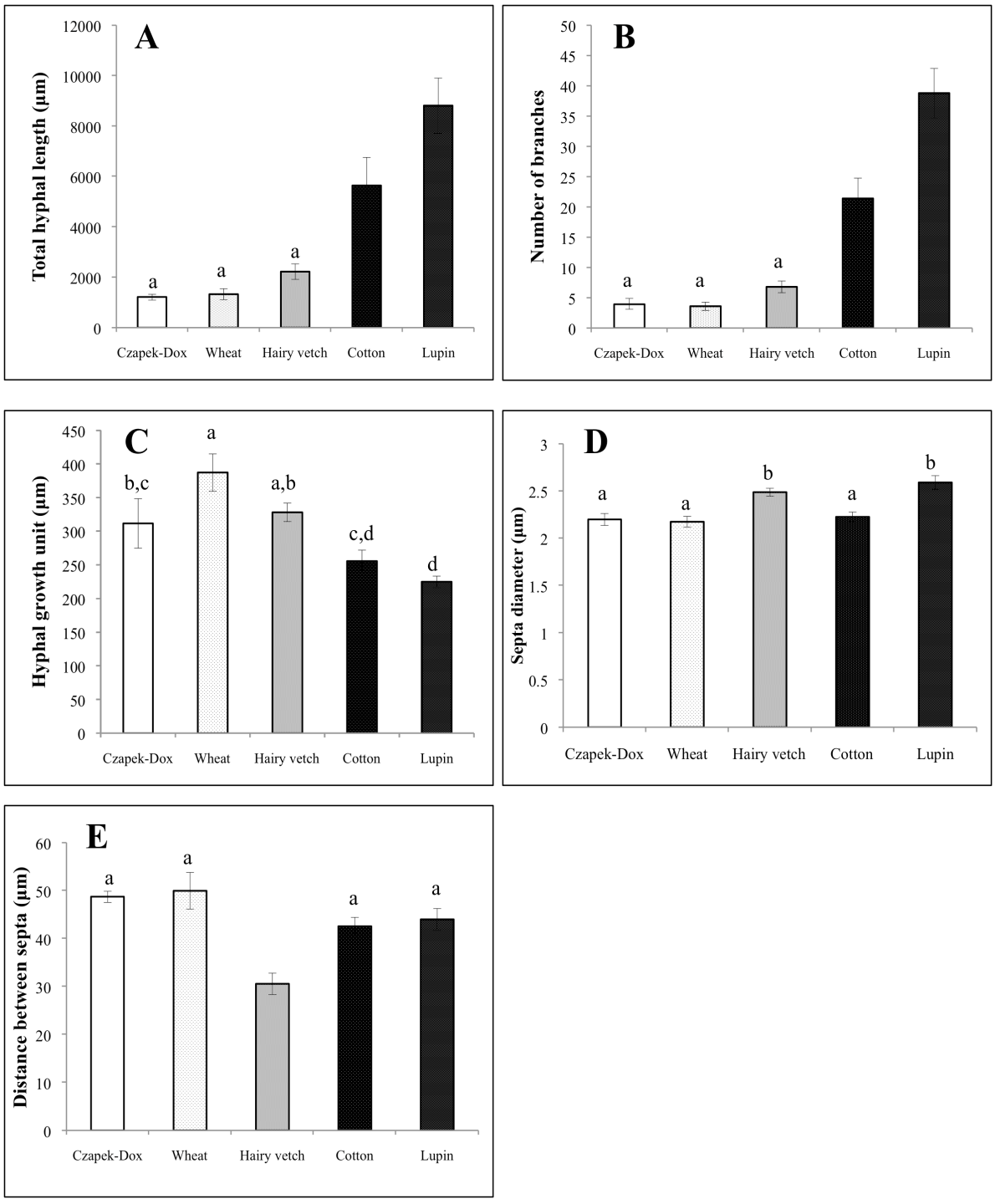

Figure 15. Quantification of hyphal morphology of *T. basicola*. *T. basicola* was grown for 24 h at 25°C in Czapek-Dox and Czapek-Dox supplemented with non-host (wheat, hairy vetch) and susceptible host (cotton, lupin) root extract (1:10 v:v dilution). **(A)** total hyphal length, **(B)** degree of branching, **(C)** hyphal growth unit, **(D)** septa diameter, **(E)** distance between septa. Columns labeled with the same letters do not differ significantly (Duncan's test ($P < 0.05$). Bars represent standard errors of the mean from 5 germinated endoconidia.

Morphological hyphal differences were observed and two important observations need to be emphasized: the strong positive influence of host root extract on total hyphal length and degree of branching and the significant decrease in length of the apical compartment observed in the presence of hairy vetch root extract. This decrease may be explained by the observed over-expression of the proteins septin, chaperonin containing T-complex protein 1 (CCT), actin, and a hypothetical protein with a BAR domain and a predicted function in cytoskeleton organisation.

In summary, our research has demonstrated that both non-host and susceptible host root extracts influence growth, colony and hyphal morphology and the proteome of *T. basicola*. Analysis of 2-DE maps of *T. basicola* revealed that the plant root extract effect depends more on the plant than the host/non-host status. Using a combination of MASCOT MS/MS ion searching, de novo sequencing and sequence similarity searches, we assigned peptide sequences to 41 differentially expressed protein spots. Based on the homology and identification of putative conserved domains in the homologous proteins identified, we suggested that vitamin B6 is important in the *T. basicola* response to hairy vetch extract. We also report a possible effect of compounds present in lupin root extract on lipid accumulation through the activity of malic enzyme and, finally, we identified putative ''morpho'' proteins, which could account for the morphological differences observed. To conclude, this is the first study to offer insight into the molecular changes occurring in *T. basicola* when in contact with host and non-host plant materials. This work lays the foundations for further studies that will aim to validate and detail the exact role of the putative proteins identified here, as well as their importance in the activities that rule this host–pathogen interaction. Acquisition of this knowledge may provide a rational basis for the development of new control strategies.

4.2. Objective 2 - Identification of plant proteins involved in cotton response to virulent *T. basicola*

Identification of cotton root proteins involved in response to *T. basicola* infection

The results of this section were published in a highly ranked international journal (J.V.F. Coumans, A. Poljak, M. J. Raftery, D. Backhouse and L. Pereg-Gerk (2009) Analysis of cotton (*Gossypium hirsutum*) root proteomes during a compatible interaction with the black root rot fungus *Thielaviopsis basicola,* Proteomics, 9 (2): 335-349).

The full information on the results of this study can be found in the published article attached to this report. A summary of the major highlights is given below.

A proteomic approach was taken to uncover the inducible molecular defense mechanism of cotton root occurring during the compatible interaction with the pathogen *Thielaviopsis basicola*. Microscopic observations of cotton root inoculated with a suspension of pathogen conidia showed that this (necrotrophic hemibiotroph) fungus interacts with the plant and completes its life cycle in our experimental system.

Briefly, analysis of the global changes in the protein expression levels in cotton was undertaken with two purposes:

(1) to observe the time course of the global protein changes occurring during *T. basicola* infection.
(2) to identify protein spots that were up-regulated or down-regulated in response to infection by *T. basicola*, as these proteins are most likely involved in the plant defence response.

Samples for proteome analysis were taken 1,3,5 and 7 days post inoculation of the cotton seedling roots with *T. basicola*. We found that over a 7 days post inoculation period the expression level for the majority of the proteins (approximately 45%) was not altered and that more plant proteins were down-regulated (32%), especially in the early stages of infection, than up-regulated (10%). Out of 900 protein spots analysed, we identified 97 protein spots with an average expression level at least 1.75 higher than those of non-inoculated (NI) samples (statistical significance level of $P < 0.05$).

Out of these, 58 spots could be analysed by mass spectrometry and identified through database search. Since the cotton genome has not been fully sequenced yet, the $NCBI_{nr}$ as well as the EST-others database were searched and when a protein sequence from the *Gossypium* species could not be confidently assigned, cross species identification was performed. The identified proteins could be classified into five major groups according to the biological process they participate in, as shown in Figure 16, and their functional significance is briefly discussed below.

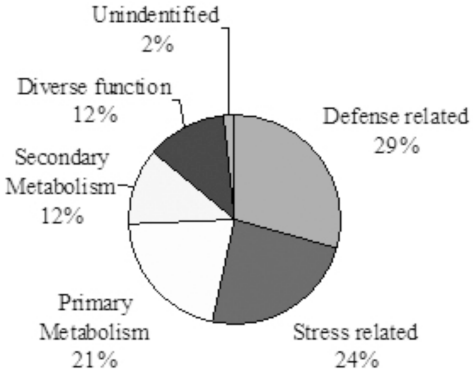

Figure 16. Functional classification of the proteins identified by mass spectrometry and found to be up-regulated in cotton root after inoculation with *T. basicola*. The relative percentages of proteins in each category are shown.

Defence responses: pathogenesis-related proteins

Not surprisingly, a number of the up-regulated proteins correspond to pathogenesis-related proteins (PR). The majority of them belong to the PR-10 family. PR-10 proteins have been shown to be induced in response to pathogen attacks, including fungal pathogens. In cotton, it was previously reported that PR-10-related genes were the most common PR genes induced in hypocotyls infected with *F. oxysporum* f. sp. vasinfectum. A gene for a putative PR-10 has also been reported to be induced in bacterial-blight resistant cotton after inoculation with *X. campestris* pv. malvacearum. While the specific function of PR-10 proteins is still unknown, some members of the family are suggested to be involved in hormone-mediated disease resistance in cotton. Other members of the PR-10 family display ribonuclease activity. It has been postulated that PR-10 proteins with RNase activity are liberated from damaged host cells and could protect the plant by acting directly on the pathogens. Another PR protein up-regulated during *T. basicola* infection is a putative thaumatin protein. Members of this family have been shown by other groups to inhibit hyphal growth or spore germination of various fungi by permeabilising the fungal membranes. The last two protein spots identified in this category correspond to a *Meloidogyne*-induced protein MIC-3, a protein reported to be induced only in cotton root following *Meloigogyne icognita* infection.

Stress responses

Six protein spots similar to putative oxidoreductase and one spot, identified as a peroxidase, correspond to enzymes that are likely to be involved in the apoplastic oxidative burst. Peroxidases are known to be induced by several stresses. In response to pathogen attack, it has been proposed that the production of ROS such as superoxide anions and hydrogen peroxide creates an unfriendly environment that could eventually reduce pathogen growth as observed by other groups in cotton infected with *X. campestris* pv. malvacearum. Moreover, peroxidases have also been associated with oxidative H2O2-mediated cross-linking of cell wall proteins, such as formation of lignin that reinforce the cell wall, as well as with cell wall deposition of phenolic compounds, thereby reducing the spread of the pathogen.

A putative benzoquinone reductase was also found to be up-regulated. This protein is known to play a key role in lignin degradation by wood-rotting fungi. Four spots corresponding to putative glyoxalase I were up-regulated. Interestingly, glyoxalase I was reported to increase in response to various environmental stress conditions in several plants and to be involved in the detoxification of cytotoxic compounds such as methylglyoxal. GST, a key defense enzyme against xenobiotic toxicity was also found to be up-regulated. Such enzymes are known to be induced by biotic stress or by treatment stimulating the plant defense reaction. While the exact function of this protein is a matter of speculation, it may protect the plant from the oxidative damage occurring during *T. basicola* infection. Finally, a putative short-chain alcohol dehydrogenase was identified. In cotton, alcohol dehydrogenases are known to be induced in roots following anaerobic stress.

Metabolism: Primary

Another major group of proteins found to be responsive to *T. basicola* infection are those involved in primary metabolism. Up-regulation of enzymes from both glycolysis and gluconeogenesis as well as up-regulation of an enzyme of the citric acid cycle and the glyoxylate cycle suggested an important role for sugar metabolism in the defence response. Identification of putative ATP synthases suggested the plants need for energy, and identification of putative cysteine synthases, a glutamate dehydrogenase and a putative ribosomal protein suggested an alteration of amino acid synthesis and nitrogen metabolism.

Metabolism: Secondary

All protein spots identified in this group are enzymes leading to the formation of isoprenoids, which include gossypol and related phytoalexins. Production of gossypol has been thought to contribute to the resistance of cotton to fungal pathogens. Phytoalexin synthesis genes were also shown by other groups to be induced during cotton infection with *F. oxysporum* f. sp. vasinfectum. However, their induction by *F. oxysporum* f. sp. vasinfectum occurred late during infection and therefore could not provide an effective resistance mechanism. In our study, expression levels of these enzymes were highest at day 1 suggesting that gossypol production is not likely to be efficient in conferring resistance to *T. basicola*.

Other Proteins

Putative 14-3-3 proteins, proteasome α subunits, an Hsp70, a putative cupin-5 and a tubulin alpha-1 chain were identified. The significance of 14-3-3 proteins in the defence response is still unclear, however it has been suggested that they may be involved in (1) the hypersensitive response through regulation of the proton pump and in (2) the control of key enzymes of the nitrogen assimilation pathway as well as (3) enzymes involved in carbohydrate metabolism through their interaction with other proteins. Involvement of the proteasome in plant defence suggested a possible initiating role in systematic acquired resistance, while the over-expression of Hsp70 may confer stress tolerance.

In summary, our research has demonstrated that analysis of the cotton proteome has provided insight into the plant response to a compatible interaction with the fungus *T. basicola*. We have shown that more proteins (around 30%) are repressed than induced (around 10%) and could identify known cotton defense responses such as induction of PR proteins and formation of isoprenoids. We identified *Meloidogyne*-induced protein MIC-3 proteins that so far were only know to be induced in *M. incognita* infected cotton and reported a stress response that may be secondary to infection as proteins identified are also known to be induced by other stress conditions. We also show that an alteration of carbohydrate and nitrogen metabolism occurs and that the proteasome may be important in the cotton defense response to this pathogen. Finally, this research is the first report of some of the biochemical changes associated with the black root rot disease and has laid the foundation for further studies including proteome sub-fractionation, with the aim of understanding this host-pathogen interaction in order to developed better control strategies for *T. basicola* infection.

4.3. Objective 3 - Progress towards understanding the response of the plant to current, successful, treatments and induced resistance in the plant

Characterisation of cotton root proteome response to acibenzolar-S-methyl treatment before and after infection with *T. basicola*.

Acibenzolar-S-methyl treatment is known to reduce he severity of black root rot and is thought to induce defence mechanisms in cotton. Proteome modifications in response to acibenzolar-S-methyl treatment were studied in this project. The first objective in this work was to design a reproducible culture medium for cotton plant that would sustain growth for three weeks in controllable conditions. This was achieved using a mix of vermiculite-perlite (1:3) containing 0.8 g/L of Aquasol soluble fertilizer (Yates).

The second objective was to evaluate if the acibenzolar-S-methyl treatment reduce the severity of the symptoms on the taproots after infection with *T. basicola*. We observed a decreased of severity of around 30% ($P < 0.02$) at a concentration of *T. basicola* of 1000 conodia/ml of plant culture medium and of around 45% ($P < 0.0005$) at a concentration of *T. basicola* of 500 conidia/ml of plant culture medium.

Interestingly, we also observed a slow down in the growth of cotton plants after treatment with acibenzolar-S-methyl, as shown in Figure 17.

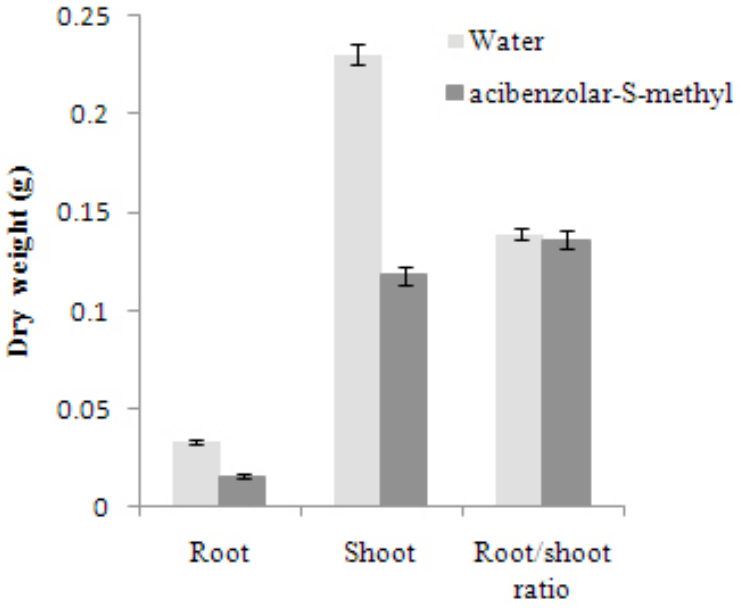

Figure 17: Dry weight of the cotton root and shoot and estimation of the root/shoot ratio after 21 days of culture.

Proteome analysis of cotton root treated by acibenzolar-S-methyl before and after infection with *T. basicola*, using 2-DE, revealed that the expression of only a few proteins is modified by acibenzolar-S-methyl treatment before infection. Analysis of the protein expression after infection with *T. basicola* revealed an increase in expression of few other protein spots, suggesting a priming effect of the acibenzolar-S-methyl. Identification of these protein spots by LC MS/MS analysis will give us a molecular understanding of the biological pathway affected by this inducer of resistance.

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.

Included below is an extract from the original application showing the three expected outputs and the corresponding expected outcomes. Each of the three will be reported on below.

Expected Outputs *eg A number of workshops are organised, asking entomologists to present & discuss findings with growers.*	**Expected Science Outcomes** (NB: A direct science outcome might not be applicable for all extension outputs.)	**Expected Industry/ Applied Outcomes** *Eg These growers gain knowledge and change practices in pesticide application.*
1. General and proteomics research is conducted for the isolation of *T. basicola* proteins involved in its interaction with plants and their characterisation.	Enhanced knowledge of the interaction of plant pathogens, which could be used for the study of other associative fungi with their host.	Following identification of pathogenicity factors, we will form collaborations with cotton breeders, who will gain knowledge on breeding cotton towards reducing the plant attraction to the fungal pathogen and/or farmers will be advised on new products for application in disease management.
2. Plant proteins involved in the association with the pathogen (stimulating or preventing pathogen infection) will be identified.	Plant proteins involved in interaction with and/or communication with microbes will be elucidated. Most interesting would be to identify proteins involved in disease resistance and/or in pathogen stimulation.	As above.
3. Upon the discovery of molecular factors controlling plant-pathogen interactions, consultation with cotton breeders and/or biotechnological companies will take place on the application of knowledge to promote disease control.		At the end of this project or in a follow-on project, a set of suggestions will be offered to industry on new ways for disease control, expectedly strategies for plant breeding (to be done by groups specialising in cotton breeding) to reduce/control black root rot and/or soil additives to target *T. basicola* pathogenicity factors.

Expected project output/outcomes 1: General and proteomics research is conducted for the isolation of *T. basicola* proteins involved in its interaction with plants and their characterisation.

As can be seen in the result section there has been a significant progress in, both, developing and utilising tools as well as developing research strategies for the study of the plant pathogen *T. basicola*. Protocols and tools for the genetic manipulation of *T. basicola* have been established and resulted in the production of a bank of fungal transformants (genetically manipulated strains). Such a bank can be used for the isolation of strains affected in any fungal property of interest – in this project we concentrated on the isolation of pathogenicity mutants and studying the fungal traits that may be related to its virulence towards cotton.

This development was followed by an analysis of the properties related to pathogenicity and the interaction of the pathogenicity mutants with the cotton host. Again – research strategies and tools developed can be adopted for use in assessing the interactions between other pathogens and their hosts.

In addition to genetic tools, proteomics tools have been developed and were very effectively used in this project to study the pathogen. The success in producing protein maps for *T. basicola* was followed by a proteome analysis and the ability to compare the response of the pathogen to host versus non-host plants and also to follow the evolution of different strains of the pathogen.

This work and the molecular tool developed can be adopted for the study of other traits of the pathogen and to be applied to both monitoring strain development (evolution) and detection of the pathogen in the soil. Such tools can be adapted to the study of other cotton pathogens.

By developing tools, strategies and generating knowledge, the corresponding **expected science outcome** identified in the application has been achieved. The interaction of the plant pathogen with the cotton host and non-hosts is better understood and both, the knowledge and the tools could be used for the study of other associative fungi with their hosts. There has been also some progress towards achieving the corresponding **expected industry/applied outcome** identified in the application. Even though the project undertaken here was a pilot project targeting the development of research tools and strategies, our understanding of the pathogen interaction with host and non-host plants and the variability of *T. basicola* strains originated from different host systems contributed to discussions with plant breeders from the CSIRO Plant Industry and we have contributed *T. basicola* strains for them to be used in their cotton breeding against *T. basicola*.

***Expected project output/outcomes 2*:** Plant proteins involved in the association with the pathogen (stimulating or preventing pathogen infection) will be identified.

As can be seen in result sections 4.2 and 4.3 of this report, the foundation has been laid and the tool developed and optimised for the identification of cotton proteins that are regulated in response to pathogenic *T. basicola* and in response to substances, which induce defence mechanisms in the plant. This project has demonstrated that analysis of the cotton proteome can provide insight into the plant response to a compatible interaction with the fungal pathogen *T. basicola*. This pilot research is the first to report some of the biochemical changes associated with the black root rot disease and has laid the foundation for further studies with the aim of understanding this host-pathogen interaction in order to developed better control strategies for *T. basicola* infection.

By developing tools to analyse cotton root proteomes, research strategies and generating knowledge, the corresponding **expected science outcome** identified in the application has been achieved. Plant proteins involved in interaction with and/or communication with microbes, including those with roles in plant defence and stress resistance, have been elucidated and moreover, the techniques required to generate more information have been established. There has been also some progress towards achieving the corresponding **expected industry/applied outcome** identified in the application, as shown above for output/outcome 1.

***Expected project output/outcome 3*:** Upon the discovery of molecular factors controlling plant-pathogen interactions, consultation with cotton breeders and/or biotechnological companies will take place on the application of knowledge to promote disease control.

Although the foundations were laid for further knowledge generation as well as some important factors in the interaction of the cotton with its *T. basicola* pathogen were discovered, more progress has to be made before the results of this study could be applied. Therefore, we have been realistic when anticipated in the **expected industry/applied** outcome identified for this output that only at the end of this project or in a follow-on project, a set of suggestions will be offered to industry on new ways for developing disease control measures. It is expected that if this project continues, utilising the tools generated in a large-scale, systematic, generation of knowledge, we could advise on strategies for plant breeding (to be done by groups specialising in cotton breeding) to reduce/control black root rot and/or on soil additives to target *T. basicola* pathogenicity factors.

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.

No changes to the Intellectual Property register are required at this stage.

Project 1.01.55 and related projects undertaken by our group targeted *T. basicola*, a plant pathogen causing the seedling disease black root rot, which is a significant threat to Australian cotton especially in cooler areas and seasons. This pilot study, utilised genomics and proteomics tools to identify molecular factors with roles in the interaction between *T. basicola* and cotton roots. We aimed at identifying genes and proteins responsible for pathogenicity, especially those involved in host-specific interactions for future development of management strategies to control the pathogen's disease cycle. 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. Understanding fungal properties and the origin of the difference between fungal strains, pathogenic and non-pathogenic to cotton, can lead to improved plant breeding strategies towards resistance to the disease.

Most of the molecular genomics and proteomics as well as phenomics tools required in this project have been developed in the seed project (1.01.21). These tools were optimised and been successfully used in this project, including in:
(1) analysing the proteomes of a collection of *T. basicola* strains with varying degrees of virulence towards cotton in order to identify proteins uniquely expressed in the cotton pathogenic strain
(2) analysing the response of the cotton *T. basicola* pathogenic strain to a collection of plants grouped into susceptible hosts and non-hosts for this strain. The aim was to identify fungal proteins required specifically for the invasion of the cotton host
(3) confirming reduced pathogenicity of five *T. basicola* mutants towards cotton and analysis of their interactions with the cotton host, in order to determine the stage of the disease cycle affected in these strains
(4) producing more pathogenicity mutants by optimising the procedure to produce *T. basicola* pathogenicity mutants, using PEG-mediated genetic transformation system, which allows an insertion of a known and easily detectable element into the genome of the pathogen
(5) genetically analysing the pathogenicity mutants using a variety of techniques
(6) testing an additional transformation method, namely ATMT
(7) analysing the response of cotton to infection by pathogenic *T. basicola* strain by utilising optimised techniques for protein extractions from *G. hirsutum* roots, protein mapping and protein identification.

There is no commercial IP generated in this project. Methodologies developed are based on published techniques, which were modified and optimised for use with cotton and with *T. basicola*. Nevertheless, there is a large amount of "know how" generated in this project, as optimising the techniques to be suitable for the specific plant and fungal pathogen studied here and the development of research strategies to answer specific questions are not only time consuming but also require expertise, in particular in the fields of genomics and proteomics.

Project 1.01.55 dealt with basic questions in the biology and ecology of the pathogens and plants involved. Therefore, there are no readily available commercialised products or procedures developed. As indicated in the application, under expected outcomes, this project generated knowledge and optimised techniques that can generate further information on genes/proteins that confer resistance of cotton to black root rot and/or fungal genes/proteins that may be targets of soil additives to suppress the disease. The main *products* of this project have been "know how', knowledge and information. Information that could lead to commercial value would most probably be based on strategies for genetic manipulation of cotton and/or on the nature of soil additives. For example, knowledge generated in this project on *T. basicola* strains, their origin and their pathogenicity towards cotton, was used in communication L. Pereg had with cotton breeders at the CSIRO Plant Industry, Canberra, regarding strategies in breeding towards resistance to black root rot.

Control mechanisms of pathogenicity could possibly be exploited in 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. Since research on pathogenicity genes of other fungi exists, the idea of utilising such information for disease suppression/resistance is not novel. What is novel is utilising such tools in a system concerning *T. basicola* pathogenicity against cotton.

Possible IP to be generated in the future if the project to continue:

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 virulent *T. basicola* developed. Soil amendments would be developed according to the fungal pathogenicity genes/proteins identified and their control measures. Plant breeding could 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.

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 generation of knowledge and successful optimisation of a large number of research tools in this project allow further investigation of the complex interactions of *T. basicola* with cotton and 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 research strategy developed 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 *T. basicola* was gathered in this project by analysing cotton proteome response to its pathogen. The ability to produce *T. basicola* pathogenicity mutants and to study the proteome of cotton roots can lead to the use of proteomics in identifying proteins with altered expression in *T. basicola* mutants. Such proteins would most likely be involved in the response of the fungus to infection by the pathogen.

Another important development is the ability to distinguish virulent stains from non-virulent strains of cotton using both, genetics tools and proteomics tools. Such tools can be further developed into a mechanism (commercial/service) to detect *T. basicola* in the soil as well as to predict whether the *T. basicola* stains found in a field are pathogenic towards cotton.

The "take home message" is that a large number of modern, basic and applied research tools have been established and the knowledge base on cotton interaction with *T. basicola* has been largely increased. There is a lot of potential in going forward with this project and making use of the genetic transformation technique and the proteomics tools to systematically generate ample of knowledge about the interactions between the cotton and *T. basicola* (as well as other pathogens). It is the time to use this potential and apply the basic knowledge and research capabilities generated in this project towards an applied outcome.

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.

Cotton seedling diseases have been reported in NSW from all fields tested. *T. basicola*, the pathogen causing black root rot in seedlings, is currently found in all fields inspected in NSW. Black root rot compromises the seedling immunity to other diseases caused by *Rhizoctonia*, *Pythium*, and *Fusarium*. The Cotton Pest management Guide for 2011-12 reported seedling mortality of 36% in NSW survey. Controlling seedling diseases would enhance crop establishment and will translate into increased crop yield.

Project 1.01.55 have had strong links to the CRDC strategic plan 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. Thus, the most obvious contribution of the developments in this project to CRDC strategies would be to enhance crop protection via present and future research into the interactions of *T. basicola* with cotton.

Four papers have been published in highly ranked international, peer reviewed, scientific journals during the life of the project. These are listed below in the next section of this report. Other publications are either in preparation or require some further data generation to be completed. With the assistance of the CCC-CRC and CRDC extension groups we hope to communicate our results in industry newspapers and magazines (e.g. Spotlight, Cotton Grower). The basic concepts in a molecular genetics and proteomics type research are difficult to convey to non-experts, therefore we require the assistance of the extension groups to bring our messages to the cotton growers. Other publications are in the form of theses, written by PhD and honours students associated with this project. We plan to keep on presenting in industry workshops such as FUSCOM and expert workshops and in the Cotton Conferences, as we have done during the life of the project (see list of publications below). We will keep on presenting and keep our group informed on recent development in relevant research fields by attending international and domestic scientific conferences.

Knowledge produced in current and future projects, the foundations laid and the tools developed in this project may be used by researchers and cotton grower groups in future 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 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 characterisation of *T. basicola* isolates and finally to (3) 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.

Future research (given availability of funding) can build on the foundations laid in this project. Plans for research in the near future include utilising the knowledge, experience and tools developed over the course of the project for:

1. systematically generating a large bank of *T. basicola* mutants affected in their pathogenicity towards cotton as well as analysing fungal proteome response to treatments, to increase the possibility of identifying an important virulence factor.
2. systematically analysing cotton proteome response to different treatments (e.g. different soil-borne pathogens) to increase the possibility of identifying important plant defence mechanisms.
3. further applying genetics tools for detection and identification of *T. basicola* in cotton growing soils.

Further genomics work will be done at L. Pereg's laboratory, which is well equipped for this purpose. Proteomics work will be done in collaboration with another group, specialised in and equipped for large-scale proteomics work, to maximise output.

Publications

A. Publications relevant to this project.

Peer reviewed articles / books

Coumans J., Harvey J., Backhouse D., Poljak A., Raftery M.J., Nehl D., Katz M.E., and Pereg L. (2011). Proteomics assessment of host-associated microevolution in the fungus *Thielaviopsis basicola* isolates. Environmental Microbiology, 13:3 576-588. (published on-line in advance of print, DOI: 10.1111/j.1462-2920.2010.02358.x)

J. Coumans, P. Moens, A. Poljak, L. Pereg and M. J. Raftery (2010). Plant extract induced changes in the proteome of the soilborne pathogenic fungus *Thielaviopsis basicola*. Molecular and Cellular Proteomics, 10:1573-1591.

Coumans, J. and Backhouse, D. (2010) Protein Analysis of Abiotic and Biotic Stress Response during Cotton Vegetative Growth. In J.k. Jenkins (ed.) The sugar Industry and Cotton Crops. NOVA Science Publisher, NY.

J. Coumans, A. Poljak, M.J. Raftery, D. Backhouse and L. Pereg-Gerk (2009) Analysis of cotton (*Gossypium hirsutum*) root proteomes during a compatible interaction with the black root rot fungus *Thielaviopsis basicola*. Proteomics 9(2): 335-349.

Publication plan for scientific journal articles

Pereg and Baker. Critical steps in *T. basicola* life cycle and its interaction with plants – a review. Possible journal: *Plant Pathology, Annual reviews in Microbiology* or similar.

Pereg, Al-Jaaidi, Baker, Mijajlovic and Backhouse. Factors determining host specificity in *T. basicola*-plant interactions leading to black root rot. Possible journal: *Plant Pathology* or similar.

Al-Jaaidi, Katz, Gentile and Pereg. Genetic transformation of the filamentous fungal pathogen *T. basicola*. Possible journal: *Molecular Microbiology* or similar.

Presentations (conference, field days, workshops etc)

Conference proceedings

L. Pereg, J. Coumans, M. Katz, D. Backhouse, S. Al-Jaaidi, G.M. Ali, J. Moulynox (2011) *Thielaviopsis basicola*-cotton interactins leading to black root rot. 5th World Cotton Conference, Mumbai, India, November 2011.

L. Pereg, J. Coumans, M. Katz and D. Backhouse (2010) Linking cotton-pathogen molecular interactions and black root rot management. Key Note Speaker, CCC-CRC Science Forum, Narrabri, Australia October 2010.

L. Pereg (2009) Black root rot: Cotton disease research at the University of New England. FUSCOM June 2009, Toowoomba, Australia.

Pereg-Gerk, L., J. Coumans, M. Katz, D. Backhouse and S. Al-Jaaidi (2007) Molecular factors involved in Thielaviopsis basicola-plant interactions leading to black root rot. 16th Biennial Australasian Plant Pathology Society Conference - Back to basics: Managing Plant Disease, Adelaide, September 23-27th.

Pereg-Gerk, L., S. Al-Jaaidi and M. Katz (2007) Molecular factors involved in Thielaviopsis basicola-plant interactions. Genetic Society of Australasia, Sydney, June 26-29th.

Theses submitted

UNE PhD candidate; to be submitted March 2012; G.M. Ali; Molecular mechanisms in the pathogenesis of cotton black root rot (Principal supervisor: L. Pereg).

UNE Honours; 2011; G. Gentile; Using polyethylene glycol-mediated transformation when identifying pathogenicity genes in Thielaviopsis basicola (Principal supervisor: L. Pereg).

UNE PhD; 2009; J. Moulynox; Bio control agents for managing black root rot in Australian Cotton (Principal supervisor: L. Pereg).

UNE Honours; 2008; R. Forbes; Molecular interactions between *Thielaviopsis basicola* and Cotton Governing the pathogenesis of black root rot (Principal supervisor: L. Pereg).

UNE PhD; 2007; S. Al-Jaaidi; Transformation of Thielaviopsis basicola to study host-pathogen interactions (Principal supervisor: L. Pereg).

UNE Honours; 2005; J, Mijajlovic; Thielaviopsis basicola interactions leading to black root rot (Principal supervisor: L. Pereg).

Reports

Braun, L. (2008) Research Highlights @ UNE - UNE joins forces with Cotton CRC to stop the rot.

B. All other publications by project team during this period.

Peer reviewed articles / books

K. Amprayn, M. T. Rose, M. Kecskés, L. Pereg, H. T. Nguyen and I. R. Kennedy (2011) Plant growth promoting characteristics of soil yeast (*Candida tropicalis* HY) and its effectiveness for promoting rice growth. Applied Soil Ecology. (published on-line in advance of print, doi:10.1016/j.apsoil.2011.11.009). http://dx.doi.org/10.1016/j.apsoil.2011.11.009

G. Krishnen, M. L. Kecskés, M. T. Rose, P. Geelan-Small, K. Amprayn, L. Pereg and I. R. Kennedy (2011) Field monitoring of plant growth promoting rhizobacteria by colony immunoblotting. Canadian Journal of Microbiology, 51(11): 914-922.

Dalton, S. J., Godwin, S., Smith, S. D. A., and Pereg, L. (2010). Australian subtropical white syndrome: A transmissible, temperature-dependent coral disease. Marine and Freshwater Research, 61:342-350.

Dalton, S. J., Harrison, M., Carroll, A. G., Smith, S. D. A., and Pereg, L. (2010). Spatial and temporal patterns of Australian subtropical white syndrome at eastern Australian reefs: host range, prevalence and progression of tissue necrosis. In 'Emerging infectious diseases: global trends, surveillance and eradication' (Ed. F. Columbus). Nova Science Publishers Inc: New York.

Cheetham, B.F., Whittle, G., Ting, M., and Katz, M.E. (2010) Interactions between bacteriophage DinoHI and a network of integrated elements which control virulence in *Dichelobacter nodosus*, the causative agent of ovine footrot. In: *Biocommunication of soil bacteria*. Soil Biology Vol. 23. Witzany, G., Ed, Springer Publishing, New York, NY, Chapter 9, pp. 237-253.

Katz, M.E. and Kelly, J.M. (2010) Glucose. In: *Cellular and Molecular Biology of Filamentous Fungi.* Borkovich, K. and Ebbole, D., Eds, American Society for Microbiology Press, Herndon, VA, Chapter 21, pp. 291-311.

Rahmanpour, S., Backhouse, D., & Nonhebel, H. M. (2010). Reaction of glucosinolate-myrosinase defence system in Brassica plants to pathogenicity factor of Sclerotinia sclerotiorum. European Journal of Plant Pathology, 128(4), 429-433.

Palanisamy, S. K.A., Fletcher, C., Tanjung, L., Katz, M.E., and Cheetham, B.F. (2010) Deletion of the C-terminus of polynucleotide phosphorylase increases twitching motility, a virulence characteristic of the anaerobic bacterial pathogen *Dichelobacter nodosus*. *FEMS Microbiol. Lett.* **302:** 39-45.

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Katz, M.E., Evans, C.J., Heagney, E.E., vanKuyk, P.A., Kelly, J.M., and Cheetham, B.F. (2009) Mutations in genes encoding sorting nexins alter production of intracellular and extracellular proteases in *Aspergillus nidulans*. *Genetics* **181:** 1239-1247.

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L. Høj, A. Cano Gomez, E. F. Goulden, L. Owens, N. Andreakis, L. Pereg, M. R. Hall (2011) *Vibrio owensii* - a pathogen for prawn and lobster larvae. Annual meeting of Australian Society for Microbiology, Hobart, July 2011.

Goulden, E.F., Hall, M.R., Pereg, L.L, and Hoj, L. (2011) Probiotic protection against pathogenic *Vibrio owensii* and *in situ* visualisation of bacterial interactions associated with the phyllosoma of ornate spiny lobster (*Panulirus ornatus*). Australasian Scientific Conference on Aquatic Health, Cairns, Australia, July 2011.

A. Cano Gomez, E. F. Goulden, L. Høj, L. Owens, N. Andreakis, L. Pereg, M. R. Hall (2011) *Vibrio owensii* - a pathogen for prawn and lobster larvae. Australasian Scientific Conference on Aquatic Health, Cairns, Australia, July 2011.

K. Amprayn, I.R. Kennedy, M. Kecskes, L. Pereg-Gerk and G. Krishnen (2009) Plant response to growth promoting Pseudomonas fluorescence: Optimised rice root proteomics. 16th International Congress on Nitrogen fixation, June 14-19, 2009, Big sky Montana, USA.

G. Krishnen, M. L. Kecskes, M.T. Rose, R. Caldwell, K. Amprayn, L. Pereg and I. Kennedy (2009) Effects of rice root exudates on growth and gene expression of Azospirillum brasilense sp 245: Preliminary results. 15th SUNFix Symposium, Sydney, Australia, June 2009.

K. Amprayn, M. Kecskes, G. Krishnen, L. Pereg-Gerk and I.R. Kennedy (2009) Colonisation pattern of pseudomonas fluorescens 1N on rice seedling roots. 8th International PGPR Workshop, May 15-23 2009, Portland Oregon.

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Ganisan Krishnen, Mihály Kecskés, Michael Timothy Rose, Lily Pereg-Gerk and Ivan Kennedy (2008) Alternative Membranes as Replacement for colony Immunoblotting**.** The 12th International Symposium on Microbial Ecology, Cains, Australia, August 2008.

Ganisan Krishnen, Mihály L. Kecskés, Michael T. Rose, Lily Pereg-Gerk and Ivan Kennedy (2008) Cost saving membrane for colony immunoblotting of plant growth promoting rhizobacteria (PGPR). 14th SUNFix Symposium, Sydney, Australia, June 2008.

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Ivan R. Kennedy, Khanokon Amprayn, Abu T.M.A. Choudhury, Rosalind Deaker, Mihály Kecskés, Ganisan Krishnen, Geraldine Mijajlovic, Sally Marsh, Lily Pereg-Gerk, Michael Rose, Rodney J. Roughley, Nguyen Thanh Hien, Phan Thi Cong, Pham Van Toan and Tran Thanh Be (2008) Optimising the role of PGPRs for sustainable yields of field crops. 8th European Nitrogen Fixation Conference, Ghent, Belgium, August 2008.

Pereg-Gerk, L., S. Godwin and S. Dalton (2007) Coral disease and microbial communities in subtropical waters of the Solitary Island Marine Park. Australian Coral Reef Society, Fremantle, October 9-11th.

Dalton, S.J., S. Godwin, L. Pereg-Gerk and S.D.A. Smith (2007) Characteristics of the coral disease Subtropical White Syndrome: mode of transmission and temperature effects. Australian Coral Reef Society, Fremantle, October 9-11th.

Theses submitted

USYD PhD; 2011; G. Krishnen; Molecular interactions between plant growth promoting organisms and rice (Co-supervisor: L. Pereg)

USYD PhD; 2011; K. Amprayn; Quantifying plant response to plant-growth promoting (PGP) organisms (Co-supervisor: L. Pereg)

UNE PhD; 2011; E. Goulden; Pathogens and probionts of ornate spiny lobster (*Panulirus ornatus*) phyllosoma (Principal supervisor: L. Pereg).

UNE PhD; 2009; X. Hou; FlcA regulation of metabolism and stress response in (the soil bacterium) *Azospirillum brasilense* Sp7 (Principal supervisor: L. Pereg).

UNE PhD; 2009; A. Padney; Microbiological and molecular factors involved in the

interactions of disease suppressive bacteria *Pantoea, Exigubacterium* and *Microbacterium* with wheat (Supervisors: D. Backhouse and L. Pereg).

UNE PhD; 2009; D. Dalton; Stressors of eastern Australian subtropical corals: Australian Subtropical white Syndrome and coral bleaching (Principal supervisor: L. Pereg).

UNE PhD; 2008; E. Farrell; The biological and genetic variation amongst 25 Australian isolates of the nematophagous fungus Duddingtonia flagrans (Principal supervisor: M. Katz)

UNE PhD; 2007; S. Godwin; The Pathology and Bacterial Ecology of Subtropical White Syndrome: A Disease of Scleractinian Corals in Subtropical Eastern Australia (Principal supervisor: L. Pereg).

UNE PhD; 2007; S. Kumar; Molecular analysis of the role of polynucleotide phosphorylase in the virulence of *Dichelobacter nodosus* (Co-supervisor: M. Katz)

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

None

Part 5 – 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.

Cotton root infection by the fungal pathogen *Thielaviopsis basicola* leads to black root rot, a significant seedling disease affecting cotton production especially in cooler areas and seasons. Cotton seedling diseases have been reported in NSW from all fields tested and *T. basicola* has been found in all fields inspected in NSW. Black root rot compromises the seedling immunity to other diseases caused by *Rhizoctonia*, *Pythium*, and *Fusarium*. The Cotton Pest Management Guide for 2011-12 reported seedling mortality of 36% in NSW survey. Controlling seedling diseases would enhance crop establishment and will translate into increased crop yield.

Management strategies to control black root rot based on cotton production practices can reduce the severity of the disease and of crop losses in some cases, however yield can still drop by up to 25-40% 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 the development of 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 host roots before rotting can occur. This suggests that there are highly specific molecular interactions between the fungus and cotton that are involved in the infection progress and allow such specificity. Identification of host-specific interactions during infection could be used in developing specific control measures against black root rot.

Methods developed by our group were utilised for investigating the interactions between the fungal pathogen and its cotton host, as well as for genetic manipulation of the pathogen and for proteome analyses of both the pathogen and cotton roots. Genomic and proteomic analysis provided evidence that in Australia, *T. basicola* originated from descendents of

single strains or groups of closely related strains associated with specific hosts. Host-specific differentially expressed fungal proteins and proteins with expression levels that could be correlated with virulence against cotton have been identified. Identification of these proteins provided insight into the biochemical diversity of *T. basicola*. Analysis of the response of *T. basicola* to host and non-host extracts, demonstrated that root extracts of both non-host and susceptible-host influence growth, colony and hyphal morphology and the proteome of *T. basicola*. Analysis of 2-DE maps of *T. basicola* revealed that the effect of plant root extract depends more on the plant species than its host/non-host status. This work indicated that vitamin B6 is important in the *T. basicola* response to hairy vetch extract. It also suggests a possible influence of compounds present in lupin root extract on lipid accumulation in *T. basicola* through the activity of malic enzyme and, finally, a role for putative ‘‘morpho’’ proteins, which could account for the morphological differences observed in *T. basicola* cultures in response to different plant extracts.

Analysis of the cotton proteome has provided insight into the plant response to a compatible interaction with the fungus *T. basicola*. Known cotton defense responses, such as induction of PR proteins, proteasome induction and formation of isoprenoids, were identified as well as stress response proteins known to be induced by other stress conditions. Alteration of carbohydrate and nitrogen metabolism also occurred in the plant in response to the pathogen. Finally, this research is the first to report some of the biochemical changes associated with the black root rot disease and has laid the foundation for further studies with the aim of understanding this host-pathogen interaction in order to developed novel control strategies for *T. basicola* infection.

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 cotton resistance or other control measures, such as soil amendments. The developments of protocols and research strategies allow research into the *T. basicola*-cotton interactions to progress. Such research could lead to discoveries towards ways of controlling the disease and thus increasing cotton yields.

For further information please contact the project leader, Dr Lily Pereg, School of Science and Technology, University of New England (Email address: lily.pereg@une.edu.au Phone: 02 6773 2708)