Molecular factors in pathogen-cotton interactions leading to Black Root Rot and disease control

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Introduction

Black root rot is a seedling disease caused by the soilborne fungal pathogen *Thielaviopsis basicola*. It is a significant threat to cotton and other crops in Australia, especially in cooler areas and seasons. The pathogen, *T. basicola*, produces thick walled spores that can survive in the soil for years (Figure 1). Although it was first detected in NSW in the 1980s it quickly spread by movement of the spores attached to foot-ware or machinery wheels. In just over a decade it has come to affect more than half of the cotton farms in southern Queensland and New South Wales. Regular disease surveys of cotton fields in NSW have shown an increase of incidence from 22% of fields inspected in NSW in 1995 to over 60% of farms surveyed in NSW in the 2000/2001 season and it was estimated that the incidence of black root rot in 2004 have reached 95% of the fields regularly surveyed in northern NSW (Allen and Lonergan 1997; Allen 2002; Nehl and Allen 2001).

The integrative research program within our group concentrates on the interactions between the pathogen and its cotton host and on biocontrol of the disease. The researchers employ a multidisciplinary approach to investigate molecular factors controlling the interaction between cotton and the fungal pathogen. The project is targeted to find out what controls the crucial steps of cotton infection by *T. basicola*, and how this information could be utilised to reduce incidents leading to the black root rot disease. Another project aims to find biocontrol agents, including antagonistic microorganisms, decoy plants and proteins with the potential to reduce the disease level in cotton fields.

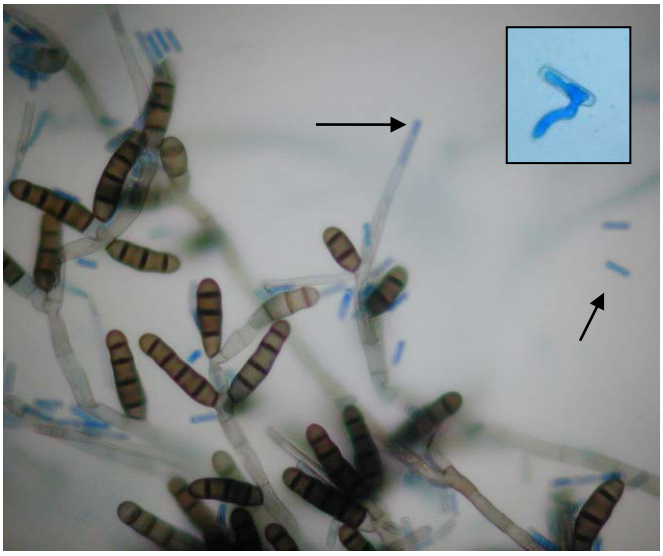

Figure 1. *T. Basicola* in a culture, showing two types of spores: conidiospores (shown with arrows) and dark, thick-walled, chlamydospores in chains. A germinating conidiospore is shown at the right top corner.

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

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

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

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

Molecular factors involved in pathogen-cotton interaction

Molecular genetic tools and proteome analysis are used in this project to study molecular factors involved in pathogen-cotton interactions. As the majority of the readers may not have background in molecular biology, we provide a short explanation on genomes and proteomes.

Living things, including us humans, other animals, bacteria, fungi and plants are all made of cells. All cells contain, among other molecules, proteins and DNA. The proteins are the machinery of the cells and also provide structural support to the cell (building blocks). The genes, contained in the DNA, are the templates for the production of proteins by the cells. So, every protein is made according to a gene/s template.

The total proteins present in the cells at a given time are called the proteome. The total genes in a cell are called the genome. Proteins can be found everywhere in the cells, free in the cell liquid or attach to structural motifs. Genes can only be found in very specific structures called chromosomes, resembling long chains of very orderly and precise sequence. Every gene on the chain has a start (start codon) and an end (stop codon). In between it has the protein template. If we disturb a gene, the corresponding protein will not be produced or will not be active. Disturbing a gene is called a mutation. A mutation in a gene/protein required for *T. basicola* pathogenicity will reduce its virulence against its host plant.

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

Generation of fungal pathogenicity-mutants

Although some progress has been made in elucidating the steps of the infection process of host plants by *T. basicola*, our knowledge of its mechanisms of pathogenesis is still limited. Genetic transformation and analysis of pathogenicity mutants has served as an efficient tool for the isolation of genes important for pathogenicity from several other fungal pathogens. A PEG (polyethylene glycol)-mediated transformation protocol (Figures 2,3), has been developed by our group for *T. basicola* mutagenesis by random insertion of plasmid DNA into the fungal genome. The plasmid DNA inserted into the fungal genome randomly disrupts genes, some of which may be involved in *T. basicola* pathogenicity. We screen for mutants with reduced pathogenicity and analyse the genes and proteins affected. Application of this technique is likely to provide knowledge on genes and proteins required for *T. basicola*-host interaction leading to black root rot and assist future studies to target resistance breeding in plants.

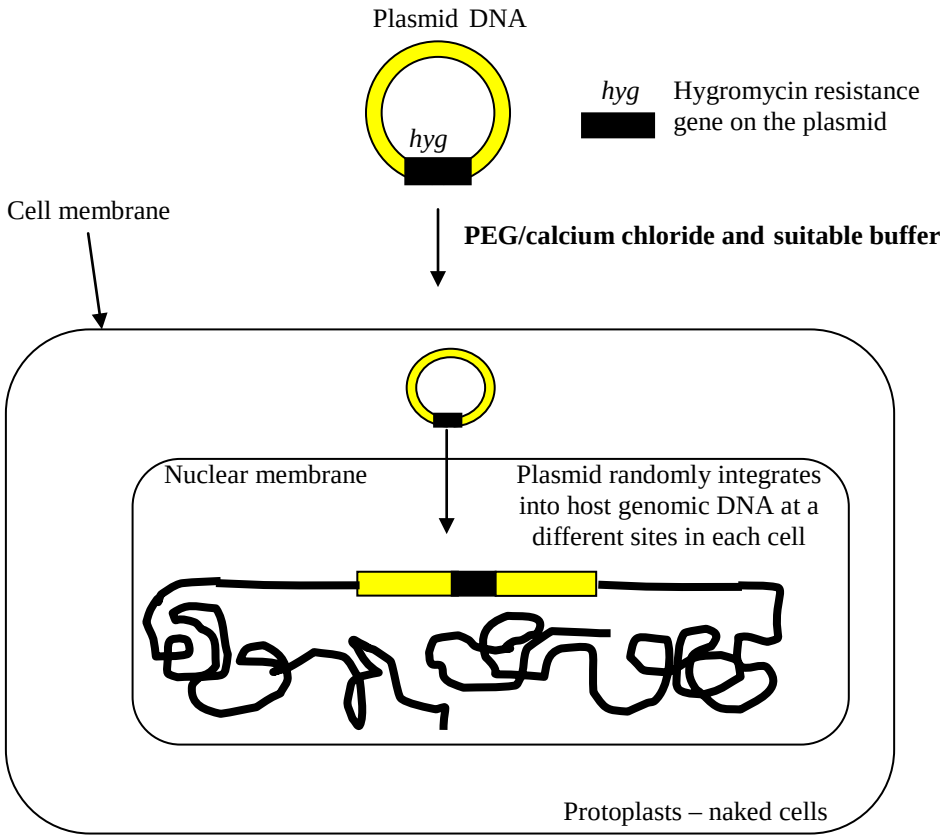

Figure 2. PEG-mediated transformation of fungal protoplasts. Random insertion of an entire plasmid into *T. basicola* genome, causing mutations.

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

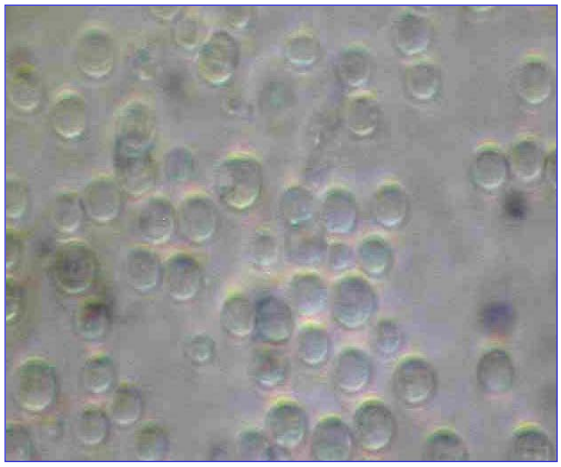

Figure 3. Protoplasts of *T. basicola*.
PEG-mediated transformation requires the production of protoplasts (naked cells, lacking cell wall) from spores, to allow plasmid uptake.

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

We achieved transformation frequencies of 1.3-2.7 transformants per μg of plasmid DNA. Our ability to grow the fungal transformants on media containing the antibiotic hygromycin (100 μg/ml hygromycin B) indicates we successfully inserted a plasmid into the genome of *T. basicola*, producing fungal transformants and possibly causing functional mutations related to the infection process. Approx. 86% of the transformants showed mitotic stability. Southern hybridization analysis of genomic DNA from 3 fungal transformants, confirmed that hygromycin B resistance resulted from random integration of the plasmid into the fungal genome.

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

Pathogenicity tests

Optimal systems for mass testing of root infection and the extent of the black root rot disease have been developed. We have been conducting two tests: one to test host infection by direct exposure of the germinating root to a fungal spore suspension (pathogenicity tests) (Figure 4) and the other testing the growth of the fungal hyphae towards the growing roots (directional growth tests).

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

Three out of the five candidates were tested for pathogenicity towards cotton in soil and were found to be less aggressive than the wild-type strain. The gene/s responsible for the reduced pathogenicity will be analysed, as well as how the gene/s may be regulated. The disrupted pathogenicity gene/s tagged by the transforming DNA will be identified using the plasmid rescue technique.

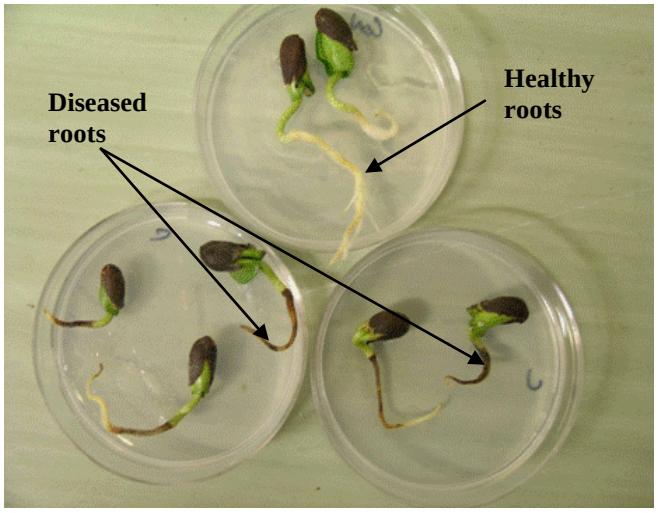

Figure 4. Pathogenicity tests on agar plates. Lower plates show seedlings inoculated by *T. basicola*, affected by black root rot. Upper plate shows control of disease-free, non-inoculated, cotton seedling.

Naturally occurring *T. basicola* isolates that vary in their host range

Germination and growth of the fungi in the presence of host and non-host plants were tested on water agar plates. As with the mutants, we performed pathogenicity tests and directional growth tests of different *T. basicola* strains towards host and non-host plants. In the absence of any plant there was no or very little germination under these conditions.

Pathogenicity tests

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

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

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

Directional growth tests

Although we could detect host specificity in pathogenicity, directional growth of all the seven *T. basicola* strains towards all of the tested seedlings was evident in a system containing seeds of one plant Vs one *T. basicola* strain. We are currently analyzing the data to see if there is a significant difference of growth rate between the seven *T. basicola* strains towards the different plants.

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

Plant seeds	***T. basicola* strain isolated from Cotton**
Cotton vs lupin	Preferred cotton
Cotton vs lettuce	Preferred cotton
Cotton vs Brocolli	Preferred cotton
Cotton vs Wheat	Preferred cotton
Cotton vs Rice	Preferred cotton

Plant seeds	***T. basicola* strain isolated from Lupin**
Lupin vs cotton	Preferred cotton
Lupin vs lettuce	Preferred Lupin
Lupin vs Brocolli	Preferred Lupin
Lupin vs Wheat	Preferred Lupin
Lupin vs Rice	Preferred Lupin

Plant seeds	***T. basicola* strain isolated from Lettuce**
Lettuce vs cotton	Preferred cotton
Lettuce vs lupin	Preferred Lettuce
Lettuce vs Brocolli	Preferred Lettuce
Lettuce vs Wheat	Preferred Wheat
Lettuce vs Rice	Preferred Lettuce

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

Comparative proteomics to detect proteins involved in pathogen-host interaction

Proteomics is used in order to study gene expression or suppression in the pathogen in response to signaling from the host plant and *vice versa*. The overall goal is to use protein mapping

(proteomics) to investigate the complex interaction between *T. basicola* and its host plants: cotton. A basic understanding of the molecular background of this interaction and its regulation at the cellular level may help in the development of effective fungal control substances that could be used in disease management.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) can separate thousands of proteins. We have established and optimized the right conditions to produce high quality protein extracts from *T. basicola* strains (Figure 5) and cotton roots, to be analysed by 2D-PAGE for protein mapping. 2D-PAGE protein maps of *T. basicola* isolates with different levels of pathogenicity towards cotton seedlings were compared to try and identify proteins involved in pathogen-cotton interactions. Cotton-isolate BRIP40192 shows high virulence, carrot-isolate 5247-6 shows moderate virulence and lettuce-isolate UQ4989 presents low virulence towards cotton tap root. Isolates 5247-6 and UQ4989 were supplied by J. Harvey and E. Aitken from the University of Queensland, where their level of pathogenicity towards cotton was determined.

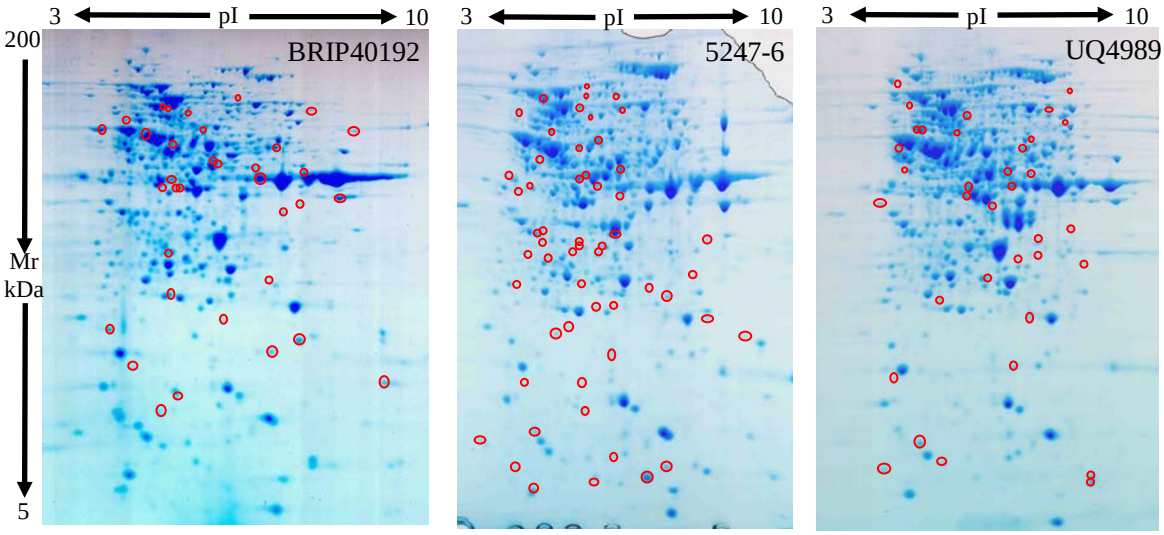

Figure 5. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of three isolates of *T. basicola* with different levels of pathogenicity towards cotton: BRIP40192, high virulence; 5247-6, moderate virulence UQ4989, low virulence. Protein spots unique to each isolate of *T. basicola* are circled.

In an attempt to identify the proteins involved in the pathogenicity, the proteomes of the three isolates grown in potato dextrose broth were examined by two-dimensional electrophoresis (2-DE) (Figure 5). Four cultures of each isolates were analyzed by 2-DE. An average of 575 protein spots per *T. basicola* strain was visualized by blue silver staining. Individual master gels were produced for each of the isolates and the gels of different strains were compared using the PDQuest software.

A common protein pattern was found consisting of a total of 343 protein spots present in all isolates. Protein spots specific to each isolates were also found (marked by circles in Figure 5) and will be analysed to see if a specific protein profiles could differentiate cotton derived *T. basicola* isolates from those isolates with lower virulence and/or with other host preference.

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.

We are currently in the process of identification of *T. basicola* proteins involved in the early events in the plant-pathogen interaction. These events are of crucial importance for the development of effective biological control strategies. Furthermore, we would then utilize pathogenicity mutants as well as the information gathered by members of our group on the pathogenicity of different *T. basicola* strains (isolated from different hosts) towards cotton and compare the protein expression maps of pathogenic and non-pathogenic strains in the presence of root exudates from cotton.

Biocontrol of black root rot

The cotton pathogen *T. basicola* does not kill seedlings by itself, but causes reduced plant vigour, which can reduce lint yield by up to 40% and make seedlings more susceptible to other cotton pathogens. As a soil-borne pathogen, it is difficult to treat using fungicides. A PhD project in our team is directed at reducing black root rot using biocontrol agents of different nature: native or introduced antagonistic soil bacteria; proteins that inhibit fungal growth; and alternative rotation crops to inhibit spore germination or induce germination in the absence of a host plant.

Bacterial control

Laboratory experiments elucidated two different bacterial cultures reducing black root rot on cotton seedlings. These bacterial cultures are currently being trialed as in furrow sprays. As *T. basicola* attacks the roots of plants, a bacterial culture is sprayed into the planting furrow before the seed is covered with soil. This coats the seed and the soil immediately surrounding it, allowing the bacteria to begin interacting with the seedling immediately and gives the bacteria a competitive advantage over native soil micro-organisms. Certain cultures trialed in the lab were applied to a field situation. Preliminary experiments with one treatment showed a significant reduction in disease severity, noted in the first 2 weeks after planting, although not full protection as was seen in the first lab experiments. Further glass house and field experiments are currently under way to examine the effect of beneficial bacterial inoculants on the level of black root rot in cotton.

References

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