Cotton Catchment Communities CRC

FINAL REPORT

(due within 3 months on completion of project)

Part 1 - Summary Details

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

BACKGROUND

Cotton seedlings are affected by a number of pathogens, many of which remain undescribed, and their effects uncharacterised. Several pathogens, including *Thielaviopsis basicola*, are currently associated with obvious declines in seedling survival and rates of growth, though the specific contribution of each to the growth of seedlings is unknown (see CRDC DAN 122C). In addition, the environment will directly influence the growth of the seedlings and each pathogen will have different effects on seedlings as the environment varies. The combined and interacting effects of pathogens and the environment on seedlings are known elsewhere as seedling disease complex. The reasons for complex effects on the seedling stage are unclear. Two factors are important: the limited extent of the root increases the whole plant effect of a limited contact by a pathogen, and the seedling has not developed a resistance response and so is potentially more vulnerable to any one pathogen. At later stages of plant growth these pathogens may have no effect because the root system is extensive and the plant resistance response has been induced.

Disease of complex aetiology is likely to require a complex response. Our overarching aim was to examine one part of the biotic component, that of microbes interacting with pathogens, with the goal of reducing the impact of the pathogens on cotton seedlings.

The question of potential pathogens in arable soils was addressed previously. We observed increased concentrations of potential pathogens in cotton soils in our molecular survey of soil microbiota (CRDC US64C), including a group loosely placed with *Rhizoctonia*, and a second group with *Fusarium*. We have also subsequently seen damage in seedlings caused by *Pythium* spp and evidence of the presence of *Rhizoctonia* and *Fusarium* spp in experimental seedlings grown in field soil from several locations. In these same molecular surveys, we observed a decline in densities of members of Trichocomaceae, amongst which are a number of species that are reported to reduce pathology associated with individual seedling pathogens.

The mechanisms of disease reduction by soil-borne fungi have been poorly characterised. In *Trichoderma*, at least five different functional aspects contribute to disease reduction, and even here, the relative importance of specific mechanisms remains unclear, and results using a single isolate continue to be unpredictable. Biological agents have enormous potential to reduce plant disease, but the means to achieve disease reduction remain uncertain. Single isolates are unlikely to be the most appropriate means of control of a disease complex of uncertain aetiology and a different approach is warranted.

Theoretically, two options remain available to attain biocontrol of pathogenic disease. The two options both consider diversity as the important contributor. The first mechanism indicates the importance in reducing seedling disease using a single biocontrol agent, this agent changes with each different pathogen and environment (Selection Effect). In the first mechanism, a diverse array of biocontrol agents is present in any one mix, but only one has an important role in each specific environment. The second mechanism views biodiversity itself as the important contributor (Biodiversity Effect). In the second scenario, it is the presence of multiple contributors that result in an additive reduction in disease. We proposed to test microbial biodiversity as a measure to reduce disease in seedlings. This approach is based on two types of evidence: the first is that isolates have biocontrol potential indicated in experimental tests, thus we should be able to provide at least one isolate active in each environment. Secondly, disease appears to be less in microbially diverse environments.

The research proposal included two themes: measurement of diversity for soil health, and testing of specific microbes to provide inoculum that contained active biocontrol agents.

1: Development of robust measures of microbial diversity and their correlation with soil health as applied to growth of cotton seedlings and land management

We obtained soil samples from across the cotton growing districts immediately prior to notification of the cessation of the project. These samples have been stored but not analysed.

2: Determine the impact of land management regime and microbial diversity on seedlings diseases including BRR in cotton

THE RESEARCH

The research in this part has two parts: laboratory measures of functional attributes, and impact of the isolates on development of disease in native soil, with particular reference to *Thielaviopsis basicola*.

A] Isolation of Potential Biocontrol Fungi.
We isolated members of the Trichocomaceae from soil collected from around the cotton growing regions of northern NSW. Isolates of other interesting fungi were made available to Chris Anderson.

B] Characterisation the Trichocomaceae
Each of the isolates of Trichocomaceae was tested for its capacity to:
1) Solubilise organic sources of Phosphate – P solubiliation by microbes in the rhizosphere may result in healthier seedlings that better resist disease. Organic P is more common than inorganic P in arable soils. Thus we tested for utilisation of organic and inorganic P.
2) Access different sources of organic energy – to survive and grow in soil, saprotrophic microbes require a source of energy, either plant exudates or cell wall materials in soil.
3) Inhibit plant pathogens – we had access to two specific pathogens of cotton, *Fusarium oxysporum* and *Thielaviopsis basicola* and we included two generalist seedling pathogens: *Rhizoctonia solani* and *Pythium coloratum*. Each isolate of Trichocomaceae was grown in liquid culture, and culture filtrate was placed in media into which each pathogen was singly inoculated. The response of each pathogen was assessed as relative growth.

C] Reduction of Seedling Disease in Field Soil by Trichocomaceae

1) Single isolates. We tested disease reduction in several ways. Soil was collected from an area of the ACRI that has particularly high levels of Black Root Rot (BRR) identified by Dr David Nehl. In order to test the potential to reduce disease we placed seed and subsequently inoculated the soil with extra *Thielaviopsis basicola* and/or each isolate of Trichocomaceae. Seedlings were harvested after 5 weeks in controlled conditions in the growth room.

2) Biodiversity – randomly selected isolates. We inoculated cotton seedlings in the field soil with *Thielaviopsis basicola* and with three, 7, 15 or 30 isolates of Trichocomaceae and grew the seedlings at 25/22C in the growth room. The experiment was repeated at 25/22C, and 22/18C. The experiment was repeated with the inclusion of green garden compost. Compost was tested as a potential carrier for spores of the Trichocomaceae.

3) Biodiversity – specifically selected isolates. We repeated the inoculation of field soil with one to several isolates of Trichocomaceae. The experiment differed from earlier experiments in three important ways: the isolates were selected for their individual capacity to inhibit plant pathogens *in soil* and the groups selected for individual inhibition of pathogens were compared to randomly selected isolates. Secondly, because we had insufficient soil from the initial collection from the severely infested field, we collected a second batch from the same field. Thirdly, because addition of *Thielaviopsis basicola* had no effect on level of BRR, we did not add extra *Thielaviopsis basicola* to the soil. The seedlings were grown for 5 weeks at 22/18C in the growth room.

4) Impact of size of seed. In early experiments, it appeared that seedlings that germinated from larger seeds were larger at harvest, even under heavy pathogen load. We tested this specifically. Seed was weighed and sorted into three groups, less than 0.95 g, between 0.95 and 1.05g, and heavier than 1.05g.

At harvest in all cases, we assessed shoot dry weight and disease caused by *Thielaviopsis basicola* as root blackening, using an index provided by Dr David Nehl. Initially we also measured proportional colonisation of roots by mycorrhizal fungi, and as data were similar to published information, we did not continue beyond the pilot.

RESULTS

A. Trichocomaceae.

We obtained approximately 70 isolates of Trichocomaceae (see Key to Identities). While nine appeared morphologically to be undescribed species, molecular sequences indicate that only two are undescribed species (Table 1). Dr A-L Markovina is thanked for assisting with the morphological identification of the isolates.

B. *In vitro* studies.

1 & 2] C & P Nutrition.

Overall, few fungi grew on cellulose, but nearly all used other cell wall constituents. Nearly all fungi solubilised organic forms of phosphate, and grew as well on organic and inorganic sources of P (see Table 1). This work was repeated with similar results and has been submitted for publication.

3] Inhibition of pathogens.

Inhibition of seedling pathogens by Trichocomaceae *in vitro* was uneven. No single isolate inhibited all four pathogens. Six isolates inhibited three pathogens, and these isolates were from several species. In addition, different isolates of one species often resulted in quite different levels of inhibition of the pathogens. Seventeen isolates of Trichocomaceae has no impact on any pathogen, and a few appeared to increase growth of some pathogens (see Table 2). The work was repeated with similar results and has been submitted for publication.

C. Disease of seedlings

Disease was measured in two ways: as seedling shoot dry weight, and as the level of disease (BRR) which is associated with *Thielaviopsis basicola,* measured as Disease Index (DI) Shoot dry weight (SDW) is relatively direct measure of seedling health, but cannot be attributed to any one pathogen or environment; the second is almost certainly associated with disease caused by *T. basicola*, but may be influenced by interactions with other pathogens present in the soil. We have not found a simple mechanism to detect disease signs or effects specifically associated with the remaining pathogens that is relatively easy to implement. In all cases, the SDW and DI were highly correlated. In the first two experiments, proportional colonisation by mycorrhizal fungi was less than 20% (low) and directly related to DI (Fig 1).

1] Single Isolates.

Note that the data presented here were collected after we started to weight seeds prior to commencing the experiment. Of the 70 isolates tested, some 20 statistically significantly increased SDW after adjusting SDW for the effect of the weight of the seed (Table 3). Slightly more fungi increased seedling growth than decreased it. Also note that five isolates significantly decreased SDW, after weights were adjusted for seed weight. That is, five Trichocomaceae fungi appear to be mild pathogens under the experimental conditions. Despite the use of eight replicates, enormous variation in seedling response was observed within any one treatment (inoculant), and the variation overwhelmed apparent increases in seedling growth. However, SDW and DI did not significantly interact, indicating that seedling growth was independent of disease in each treatment.

2] Biodiversity: Pilot experiment and temperature.

This issue was explored with a number of experiments. In the first pilot experiment, we added three, seven, 15 or 30 randomly selected Trichocomaceae to naturally infested soil also inoculated with *T. basicola*. The pilot experiment was run at 25C day temperatures, 22 night (25/22C). The results (Fig 2) indicated that presence of any Trichocomaceae reduced BRR and SDW to the level found prior to addition to soil of *T. basicola*. However, note the variation within the data. The variation indicated that some factor other than additional *Thielaviopsis basicola* influenced the shoot dry weight.

The experiment was repeated. This time we compared disease reduction and shoot dry weight at two temperature regimes 25/22C and 22/18C (Fig 3). On this occasion, disease reduction was only observed when the seedlings were grown at 22/18C, and not at 25/22C.

When compost was added to the soil prior to planting, the SDW of all seedlings was less than the absence of compost.

3] Biodiversity – Selected isolates

When we compared isolates selected from Table 3 and randomly chosen isolates the SDW and DI were not statistically different (single isolates Fig 4, four or 8 isolates together Fig 5). Because of time constraints, this experiment has not been repeated. However, we found that the natural level of infestation by *T. basicola* was approximately one one hundredth of the count in the initial experimental soil.

4] Impact of seed size.

We weighed all seeds used in the final two experiments, though data for only the last experiment is presented here. Seed size had a statistically significant positive effect on both seedling SDW (Fig 6) and negative effect on DI (Fig 7). That is seedlings from heavier seed were larger at harvest and had less BRR. We thank Dr Michael Stewart for providing statistical support that enabled the separation of the effect of seed weight in these experiments.

DISCUSSION

The results are an exciting indication that members of Trichocomaceae have the potential to reduce seedling disease of complex aetiology in naturally infested field soils. Seedling disease is caused by an unknown number of pathogens and environmental factors interacting: the situation is complex and not well understood. It is extremely encouraging that we were able on occasion to reduce disease without knowing which specific pathogens were present. Further the association between seed size and seedling disease indicates one direct mechanism for reducing seedling disease that might be considered by plant breeders and growers in the short to medium term.

The remaining mechanisms are unclear. For example, comparison of Tables 2 and 4 indicate that some pathogens may have been inhibited by the specific member of Trichocomaceae. Three Trichocomaceae (Asp 6, Pen 48 and Pen 35) significantly increased SDW, and inhibited three of the four pathogens in vitro. In addition the three fungi reduced BRR to less than 50% of the root, in comparison to the control at 62%. However, inhibition of pathogens is clearly not the only mechanism. Of the twenty fungi that increased SDW when seedling were grown in field soil, three of the fungi inhibited three pathogens in vitro, six inhibited two, nine inhibited one and one inhibited none. Of the fungi that reduced SDW, two inhibited one pathogen in vitro and two inhibited none.

Of the four pathogens tested *in vitro*, we did not find one member of Trichocomaceae that inhibited all pathogens. This result is likely to be common across all potential biocontrol agents. The consequence of this feature is that when one pathogen is suppressed, other potential pathogens may start to cause serious disease, much as has been found for pathology of the adult plant. The importance of using multiple agents of biocontrol where suppression can be maintained across many different potential pathogens cannot be overemphasised. The difficulty is to identify which microbes and in what combination are required for disease reduction in most situations and occasions.

Like other programs where disease control by biological agents is examined, we found huge variation within and between experiments. The reason for the variation is partly explained by the effect of seed size on seedling growth and BRR. Large seeds tended to form large seedlings with less BRR. It is unclear whether the same correlation applies to disease caused by other pathogens. The variation may be associated with a number of other factors, including variation in pathogen load, pathogen identity, seedling nutrition etc. We lacked any understanding of the level in seedling or soil of pathogens other than *T. basicola*. *T. basicola* was unevenly distributed, within soil across one field each season, and from season to season, and the other pathogens are likely to be similarly unevenly distributed.

Seedling size is a proxy for seedling health. Seedling size is determined by many factors that were not controlled. For instance, arbuscular mycorrhizal fungi increase seedling uptake of P, Zn, Cu and K from soil and the increased uptake may increase seedling size where soil stores are depleted. BRR was negatively correlated with proportional colonisation of roots by AM fungi, thus indicating that seedlings with BRR will have two causes of their small size: possible lack of P due to reduced AM in the roots, and the direct reduction of the development of the root system due to the pathogen. Seedling nutrition is also associated with other soil microbes. All the Trichocomaceae may solubilise P from organic and inorganic sources in soil, making that P available to the seedling. This increased supply of P would benefit the seedling if the Trichocomaceae also spread along the root system. Spread along roots and through the soil by individual Trichocomaceae was not examined. Few of the isolates had the capacity to use cellulose, so any spread would have to be along the root surface where nutrients are expressed by the seedling.

Disease may be reduced when the plant's resistance response has been induced. At least one member of Trichocomaceae can induce a host response to *Fusarium oxysporum*. It is likely that other pathogens will also be affected. We did not have time to test induction of disease resistance. However, we would predict that induction of host resistance will reduce the development of disease in seedlings.

Finally, *Trichoderma* has been shown to reduce disease following the initiation of endophytic colonisation of the root. Members of Trichocomaceae may also colonise root tissue. Again, we did not examine endophytic colonisation, or spread of the inoculants in these experiments. The mechanisms of disease reduction remain to be explored.

Part 4 – Final Report Executive Summary

Black root rot caused by *Thielaviopsis basicola* has become a major seedling disease in cotton soils since 1989. The role of seedling pathogens has become increasingly evident in the establishment and early growth of cotton. It is now clear that seedling disease has complex aetiology, many potential pathogens and environmental factors contribute to reduced seedling growth and development. This project set out to examine two aspects with a view to reducing seedling disease: (1) develop measures of diversity of microbes in soils and correlate them with soil health esp in relation to seedling disease, and (2) determine the impact of land management and microbial diversity on the development of seedling disease, especially black root rot (BRR) which is caused by the fungus *Thielaviopsis basicola.*

The project was terminated before completion of the field elements.

We were able to show that seedling disease and BRR could be experimentally reduced by the presence of some members of the Trichocomaceae (*Penicillium* and *Aspergillus*) in naturally infested field soil. Individual members individually reduced seedling disease, and on some occasions multiple randomly selected groups of these fungi reduced seedling disease including BRR. The specific reasons for variation in seedling response have not been fully clarified, but they include the size of the seed and reduced mycorrhiza in roots. Seedlings that grew from larger seeds had less disease. In addition, BRR and the proportion of root colonised by AM fungi were inversely correlated. Thus diseased seedlings were also less likely to receive minerals via their mycorrhizal fungi.

In laboratory experiments, extracts from various Trichocomaceae inhibited from zero up to three, but never all four pathogens tested. Inhibition was partial to complete, indicating the possible presence of many different inhibitory compounds or their concentration in the culture solutions. As Trichocomaceae all appeared to solubilise P from complex sources in soil, presence of these fungi may alleviate the P stress of seedlings.

Overall, the data support the hypothesis that seedling disease may, at least in some environments, be reduced in the presence of communities of microbes, specifically those shown to inhibit some of the pathogens. The suppression of BRR is particularly interesting because growers have few options to reduce this problem. Specific mixtures of Trichocomaceae, and other microbes remain to be clarified.

An immediate response to seedling disease is to consider the selection of larger seed. Seedlings from larger seeds were themselves larger. However, the effect on lint production or quality is unknown.