Australian Government
Cotton Research and Development Corporation

FINAL REPORT 2016

For Public Release

Part 1 - Summary Details

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

CRDC Project Number: 1624

Project Title: A predictive diagnostic test for *Thielaviopsis basicola* causing black root rot in cotton soils

Project Commencement Date: 01/05/2016 **Project Completion Date:** 31/10/2016

CRDC Research Program: 1 Farmers

Part 2 – Contact Details

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Signature of Research Provider Representative: Dr Maria Manjarrez

Date Submitted: **02-11-2016**

Part 3 – Final Report

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

Background

1. Outline the background to the project.

The importance of black root rot in cotton has increased to the point that it can determine if a field could be planted or not with the crop. There had been some attempts to use a predictive method to relate the levels of the fungus in the soil with the severity of the disease. However, certain correlations between soil types, history of the diseases in the paddock and quantification of the pathogen need to be tested in Australian soils. "Traditional" testing methods such as ELISA and colony enumeration methods had shown certain correlation with the disease incidence. New methods such as qPCR had also shown promising results. A mix of different testing techniques can have the possibility of yielding a method that can serve as a predictive tool without being expensive or impractical. After a comprehensive worldwide literature review, the most practical method in terms of value-for-money, practicability and availability would be set up under laboratory conditions to test the correlation between propagules of black root rot in the soil and history of disease development in the plant.

Objectives

2. List the project objectives and the extent to which these have been achieved, with reference to the Milestones and Performance indicators.

i. Development of a quantitative diagnostic method (test) with predictive potential up to trial stage

Methods

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

After a comprehensive survey of worldwide scientific literature related to detection and quantification of black root rot of cotton (*Thielaviopsis basicola*), a plate-based method used by the Extension Service of The University of California was the one showing the most promising results. An improvement to the method included DNA sequencing of some colonies to confirm the species. The method included plating soil in a specific black root rot medium, counting colonies after 30 days to try to relate number of colonies with presence of the disease and DNA identification of colonies suspected of being *Thielaviopsis basicola*. There was no attempt to re-inoculate soils and or plants with any of the pathogens isolated as *Thielaviopsis* to test for pathogenicity in cotton seedlings as that was not the aim of the project.

We tested 3 sites with "Nil BRR", 3 sites with "High BRR" and one site each for soils with a few different crop rotations such as faba beans, cotton and wheat. Each site was replicated 3-4 times when plating. After obtaining fungal growth (≈ 24 days), the most common fungi from each site (all replicates were accounted for) was sent for DNA sequencing to obtain species identification (D2 region).

Results

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

Twelve soils with different black root rot history were sent to Microbe Labs Australia for testing. In an attempt to relate black root rot incidence history with quantification in the lab we used soils with (High BRR) and without (Nil BRR) incidence of black root rot, and soils with different crop rotations, which were supposed to have different degrees of incidence of the disease. After 30 days of incubation in the selected media, plated soils showed differences in the number and type of colonies detected.

From the selected media we isolated very few "undesired" fungi. From "Nill BRR" labelled soils, the most common fungus (after DNA identification) was *Clonostachys catenulatum*/*Clonostachys rosea*, which is a fungus that had been used in the past as a broad-spectrum biocontrol agent. No *Thielaviopsis* colonies were found in this soil (Figure 1).

From soils labelled as "High BRR" (3 different sites with 4 plates replicated in each site) the number of colonies varied in each replicate, which was probably due to differences on the depth and time of sampling. In general, the mean number of colonies in "High BRR site 1" was 24 colonies/g of soil, zero colonies in "High BRR site 2" and 224 colonies/g in "High BRR site 3". See Figure 2, Table 1.
In addition to *Thielaviopsis*, a high number of *Alternaria alternata* and *Cylindrocarpon destructans* were also isolated from "High BRR" soils. In some cases, these fungi were as common as *Thielaviopsis*; in "High BRR site 2" the mean number of colonies was 48/g soil (Table 1).

In the soils with different rotations, the number of *Thielaviopsis* was highly reduced when compared to the "High BRR" soils, with the lowest in wheat and faba beans rotations and the highest in cotton-cotton rotation. See Table 2. However, in these soils the number of *Alternaria alternata* and *Cylindrocarpon destructans* colonies were high, ranging from 45 to 55 colonies/g of soil.

To finish, soils before and after flooding for 36 days were also incubated using the same method and the results are listed in Table 3 and Figure 4. From there it can be concluded that the test was able to differentiate between both treatments, with the total number of fungal colonies found to be lower in the treatment after flooding (44/g soil) when compared to the treatment before flooding (96/g of soil). The results also showed a reduction on *T basicola* before and after the treatment (5 vs 21.6 colonies/g soil, Table 3).

Figure 1 Plates showing colonies after "Nil BRR" soil was incubated for 24 days. Smaller, dark-grey colonies are Alternaria alternata. No *Thielaviopsis* colonies were isolated from these soils. Small, creamy colonies are *Clonostachys catenulatum/Clonostachys rosea*.

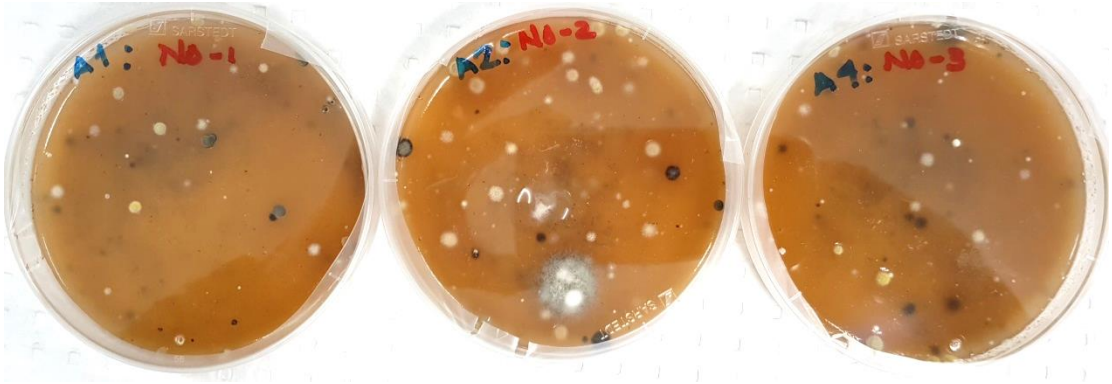


Figure 2 Plates showing colonies after "High BRR" soil was incubated for 24 days. Black colonies are *Thielaviopsis basicola*. Smaller, dark-grey, colonies are Alternaria alternata. Big, white fluffy colonies are *Cylindrocarpon destructans*. The number of colonies per gram of soil varied in each sample from nil to 224.

Table 1 Average number of colonies (3 replicated plates) of black root rot recovered per gram of soil with ("High BRR") and without ("Nil BRR") symptoms of the disease. Other fungi could include *Alternaria alternata*, *Cylindrocarpon destructans* or Clonostachys *catenulatum/Clonostachys rosea.*

	Thielaviopsis basicola	**Other Fungi**
Nil BRR-1	4	78
Nil BRR-2	1.3	213
Nil BRR-3	0	9
High BRR-1	23	82
High BRR-2	12	48
High BRR-3	220	16

Figure 3 Plates showing colonies after soil from different rotations were incubated for 24 days. The number of Thielaviopsis per gram of soil varied with the rotations.

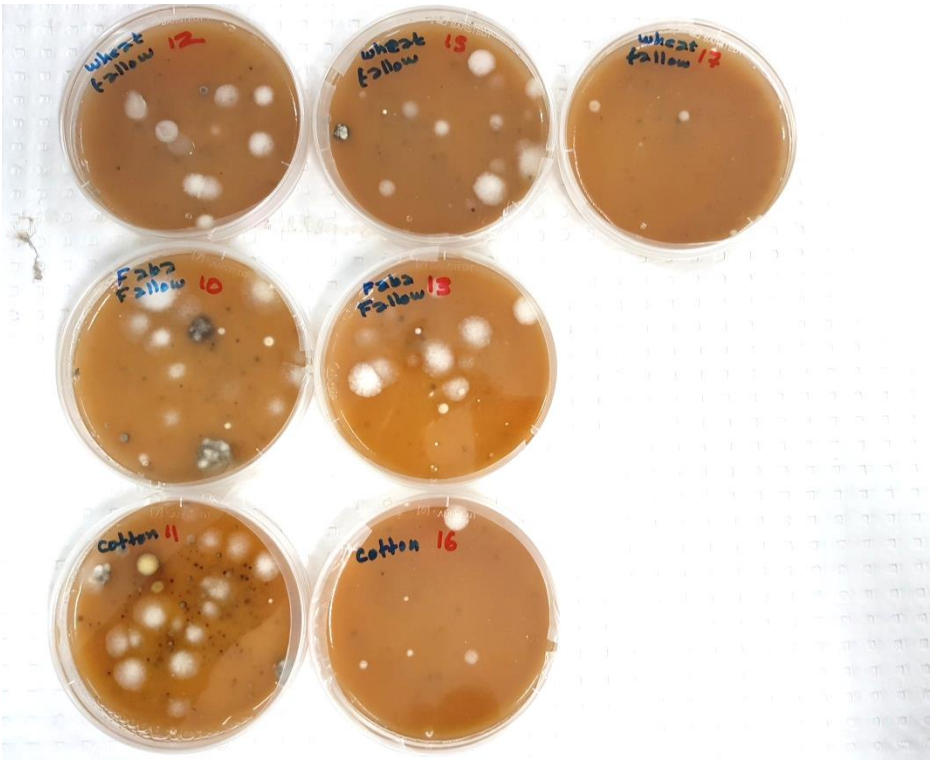


Table 2 Average number of colonies (3 replicated plates) of black root rot and other fungi recovered per gram of soil. Number of colonies varied depending on the crop rotations. Other fungi could include Alternaria alternata, Cylindrocarpon destructans or Clonostachys catenulatum/Clonostachys rosea.

	Thielaviopsis basicola	Other Fungi
Wheat Fallow	15	50
Faba-fallow	10	42
Cotton-cotton-	45	55

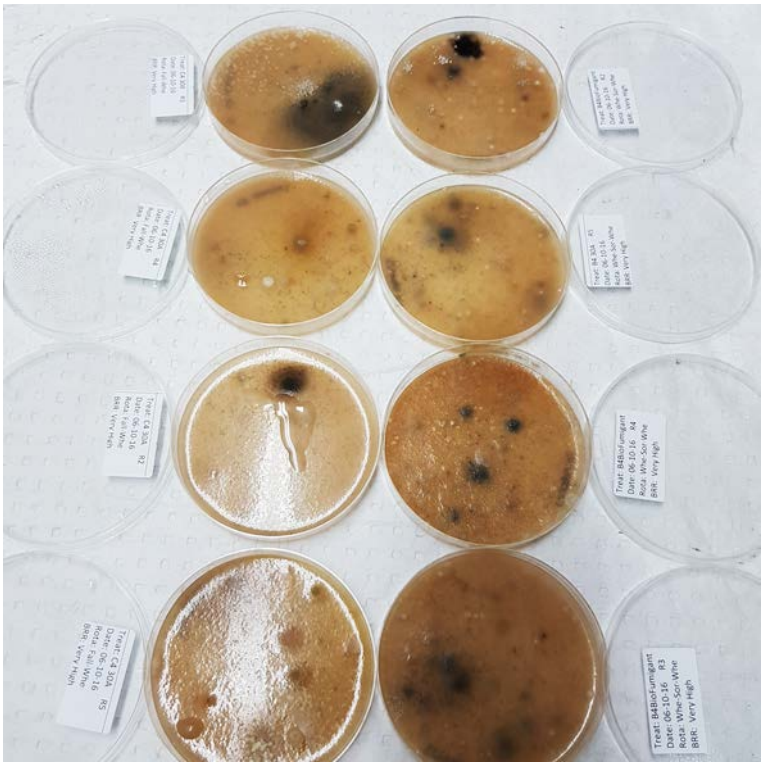
As mentioned above, cotton seedlings were not used in this project. Testing inoculum densities against incidence of the disease in the field as a way of cross checking for the reliability of the method developed here needs to be carried out. The sole aim was to develop a method capable of detecting pathogen populations. Based on the results, the plating method used in conjunction with DNA sequencing was capable of detecting certain black root rot levels under different agricultural management.

In addition, the interaction between *Alternaria alternata* and *Cylindrocarpon destructans* with *Thielaviopsis basicola* needs to be addressed if a more reliable testing tool is needed.

Table 3 Average number of colonies (6 replicated plates) of black root rot and other fungi recovered per gram of soil before and after flooding the soil for 36 days. Other fungi could include Alternaria alternata, Cylindrocarpon destructans or Clonostachys catenulatum/Clonostachys rosea.

	Thielaviopsis basicola	Other Fungi
B4-30-11-15 before flooding	21.6	44
C4 30-11-15 after flooding	4.5	96

Figure 4 Plates showing fungal colonies before (plates on the right) and after (plates on the left) soil was flooded for 36 days. The number of Thielaviopsis per gram of soil varied according to the treatment.



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.

So far the plating method used in conjunction with DNA sequencing was capable of detecting certain black root rot levels under different agricultural management, which could be just enough to help cotton producers to take more informed decisions before planting. However, inoculum densities of *Thielaviopsis basicola* against severity of the disease in the plant needs to be addressed.

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.**

There was no commercially significant development yet as fine tuning of the method would require further investigation including glasshouse and field trials. A further lab replication of the results presented here need to be completed and is in progress.

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 method for the detection of *Thielaviopsis* populations under different management strategies may be implemented after further replication of some of the lab work. The test may be used in first instance to test for "artificially" infested soils under glasshouse conditions with further application to disease severity in field soils. In addition, the method was able to detect a probable interaction among other pathogens (Alternaria, *Cylindrocarpon*) and *Thielaviopsis*, which had not been considered as important before.

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.
   a) The test method needs to be fine-tuned using "artificially" infested soils under glasshouse conditions firstly, and under field conditions secondly. Once inoculum densities are successfully surveyed, history of the paddock and/or number of years with cotton plantation also needs to be related to the severity of the disease.
      The interaction between other plant pathogens and *Thielaviopsis* needs to be addressed to really be able to plan management practices ahead.
   b) The method needs broader assessment as physical and chemical soil characteristics would impact the severity of the disease. Educating farmers on the different interactions between soil pH, soil structure, soil carbon, etc and disease severity is a must for successful planning management. Cotton producers need to be aware that testing their soils is just the introduction of better practices management for cotton.
   c) Isolation of *Thielaviopsis* from infested soils to create "artificial" inoculum, which in turn can be used to re-infect cotton seedlings with the aim to test inoculum levels against severity.
      The only way to test for the interaction between other plant pathogens and *Thielaviopsis* is isolating those fungal pathogens, creating artificial inoculum and testing the interaction with *Thielaviopsis* by infecting cotton seedlings with and without black root rot. The results may be used to design a multi-species detection test.

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

No formal publications were produced, except for a protocol on how to sample.

1. "Soil sample procedure for black root rot -BRR- in cotton fields". Sampling instructions on how to take samples for black root rot analysis. For farmers use.

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

No online resources were published.

Part 4 – Final Report Executive Summary

A predictive diagnostic test for *Thielaviopsis basicola* causing black root rot in cotton soils

After a comprehensive survey of worldwide scientific literature related to detection and quantification of black root rot on cotton (Thielaviopsis basicola), a plate-based method used by the Extension Service of The University of California was the method showing the most promising results to be used in Australian soils. However, an improvement to the method was needed, and it came in the form of DNA sequencing of the isolated colonies that grew after 30 days of incubation, that allowed for the detection of black root rot levels under different agricultural management practices. The test, which may be capable of detecting inoculum levels that could cause severe disease symptoms, is intended to be use as a tool to help cotton farmers to make informed decisions before the planting season. So far, by using this method, we were able to separate soils that were showing high levels of the disease against those that were not showing any symptoms. A useful threshold was set up to indicate when a soil may allow to crop cotton or better use a rotation. However, there were soils that showed high levels of black root rot, which did not present high levels of *Thielaviopsis* basicola but rather other pathogens such as Alternaria and *Cylindrocarpon*, indicating that black root rot may be the result of more than a single pathogenic species. The soils screened so far also included flooded soils and soils under different crop rotations. However, a clear trend between different agricultural practices cannot be drawn without using several sites and replicates and different degrees of infective propagules to recreate levels of severity in glasshouse and in the field. The development of this "predictive" test may be used to help farmers with pre-plant decisions. However, the use of this tool is only one step out of the entire management practices pool that may interfere with the severity of the disease.