SUMMER SCHOLARSHIPS 2005/2006 SEASON

FINAL REPORT

PROJECT TITLE

Protection of cotton seedlings from *Thielaviopsis* by microbes

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PLAIN ENGLISH SUMMARY

TITLE

Protection of cotton seedlings from *Thielaviopsis* by microbes.

STAFF

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AIM

This project examined the interaction between soil microbes and the cotton root pathogen *Thielaviopsis*.

PROJECT SUMMARY

Thielaviopsis basicola causes black root rot of cotton. It was first reported in Australia in 1989 and is now widespread. No commercial cotton cultivars are resistant to black root rot and control of the pathogen is difficult. This project investigated the impact of soil microbial diversity on the black root rot pathogen.

Comparison of disease severity in cropped and uncropped soils, artificially infested with *T basicola*, failed to show suppression of disease under conditions of higher microbial diversity. Similarly introduction of native microbes into sterile soil did not decrease disease severity in the presence of the pathogen. However individual screening of microbes demonstrated that some microbes are capable of suppressing the pathogen under controlled conditions, indicating that the presence of functional groups of microbes may be more important than simply species diversity.

CONTENTS

1 INTRODUCTION

Thielaviopsis basicola (Berk. & Br.) Ferraris, is a root pathogen of many economically important plants including cotton (*Gossypium hirsutum* and *G. barbadense*). Black root rot of cotton caused by *T. basicola* was first reported in Australia in 1990 (Allen, 1990) and is now found on 97% of farms surveyed annually in NSW (Nehl et al, 2004). Severe black root rot delays crop maturity and causes yield loss (Allen, 1990). No commercially available cultivars are resistant and management of the disease is difficult.

Modern agriculture involves large scale human intervention in the environment, diverse and complex ecosystems are replaced by monoculture and biodiversity is greatly reduced. Monocultures are susceptible to devastation by antagonistic organisms. Crop protection practices aim to exclude unwanted organisms from the local environment using chemical and cultural methods. However crops are increasingly being viewed as functioning ecosystems that need careful management with, for example, awareness of the role of mycorrhizal fungi in crop growth and the importance of soil biology (Feng et al, 2002; McGee et al, 1999).

The relationship between biodiversity and ecosystem functioning is rapidly becoming a major field of biological research. Experimental and theoretical ecology are beginning to look at how biological diversity affects ecosystem processes. Ecosystem functioning is of economic significance to industries such as agriculture. Ecologists and agricultural scientists are beginning to ask whether depauperate ecosystems function differently or perform less efficiently than more biodiverse ecosystems (Lareau, 2000; Ehrlich & Wilson, 1991; Chapin, et al, 1998). The question of how biodiversity affects ecosystem functioning is of central importance to the future of agriculture. Ecosystems with greater biodiversity may be more resistant to devastation by new pests, diseases or climatic processes. Diversity of soil microbes may protect ecosystems from epidemics of soil borne diseases. Greater microbial diversity increases the number of interspecific interactions such as competition and antagonism. Microbial antagonists in species-diverse soils may protect crops from soil borne pathogens.

Pathogen-suppressive soils have been observed for many soil borne diseases (Sturz, et al, 1997). The suppressive natural of soils is often biological and transferable. Biological

antagonism includes fungistasis, niche competition and antibiosis (Sturz, et al, 1997). Fungistasis, the inhibition of spore germination, has been shown to be strongest in soils with high microbial activity and organic content (Chinn 1967) and soil bacteria have been implicated (Epstein & Lockwood, 1984; Franklin & Patrick 1985). Saprotrophic fungi and bacteria in soil can act as a sink for growth-limiting nutrients, thus competitively suppressing pathogens (Suslow, 1982; O'Sullivan & O'Hara 1992). Soil saprotrophs can also produce anti-fungal metabolites that act in direct antibiosis (Sturz, et al, 1997).

Members of the fungal family Trichocomaceae, which includes the major anamorphic genera *Penicillium* and *Aspergillus*, are known to produce antimicrobial secondary metabolites (Pitt, 1994). Many species of fungi isolated from the root zone of cotton have activity against *T. basicola* including many species of Trichocomaceae (Tashlieva, 1980). Species of Trichocomaceae are ubiquitous in the Australian environment (McGee, et al, 2006) but soil ecosystems are complex and diverse. In order to investigate the impact of soil microbial diversity on *T. basicola* in cotton crops it is necessary to limit the diversity of microbes studied. The Trichocomaceae, because of their demonstrated antimicrobial function and ubiquitous distribution, are a suitable group for initial investigation.

An investigation of the role of members of the Trichocomaceae in crop soils and their impact on the black root rot causing pathogen *Thielaviopsis basicola* will aid in the understanding of biodiversity in crop pathogenesis. A better understanding of the relationship between soil biodiversity and crop pathogenesis is necessary for developing methods of managing crops as ecosystems for efficient and productive agriculture.

2 MATERIALS AND METHODS

2.1 Isolation of Trichocomaceae from soil and cotton plants

Soil

Seven soil samples were collected from a range of habitats around Narrabri on the New South Wales north-west slopes and plains (see appendix for details). Ten grams of soil from each sample was suspended in 90 mL of a sterile 1% agar solution by shaking with a mechanical shaker for 15 minutes. A dilution series for each sample was produced by mixing a 1 mL aliquot of each suspension with 9 mL of sterile water and repeating four times. For each dilution a 200 µL aliquot was spread on the surface of Petri dishes containing either dichloran rose Bengal chloramphenicol (DRBC) agar, dichloran glycerol 18% (DG-18) agar or potato dextrose agar (PDA). Over a 7 d period the spread-plates were examined under a low power dissecting microscope and colonies of interest subcultured onto PDA.

Cotton tissue

Leaf, stem and root samples were taken from five cotton plants growing in soil infested with *T. basicola* at the Australian Cotton Research Institute (ACRI field Old 2, 30º12.296'S 149º35.580'E). Sections of an apical leaf, a basal leaf, stem and tap root were surface sterilised for 60 seconds in a 1% sodium hypochlorite and 5% ethanol solution and placed on the surface of DRBC agar plates. For 7 d the plates were observed under a low power microscope and any *Trichocomaceae*-like colonies subcultured onto PDA.

All cultures were maintained on PDA and subcultures were sent to Sydney for identification and molecular analysis, and to be added to the University of Sydney collection.

2.2 Comparative soil experiment

Soil collection

Cultivated and non-cultivated soil was collected using sterile implements. Cultivated soil (R1) was collected from the furrow of a cotton crop (ACRI field R1, 30º12.705'S 149º35.030'E). Non-cultivated soil (NR1) was collected from the adjacent stock reserve (30º12.714'S 149º34.999'E) underneath native vegetation, to simulate a natural soil with similar chemical and physical properties. Thirty 700 mL milkshake cups with six holes in the bottom were filled with each soil and a further 30 with a 50/50 mixture of both soils.

Preparation of Thielaviopsis *inoculum*

Thielaviopsis colonies were grown on PDA for 7 days. Three colonies were grown on each of five PDA plates. Approximately 1 mL of sterile distilled water (SDW) was added to the surface of each agar plate and the surface gently scraped with a glass microscope slide. The liquid was then poured through a 106 µm sieve to remove hyphal fragments. The mixture was centrifuged at 3000 rpm for 3 minutes and the supernatant discarded. The spores, approximately 0.5 mL were resuspended in 100 mL of distilled water. A 1 mL aliquot was taken for counting. The remaining 99 mL were sprayed onto 810 g of quartz sand (Sigma) using a household spray bottle and left to dry. The final sand-inoculum mix carried 143,000 spores per gram.

Seedling germination

Folded paper towelling was placed in the bottom of a rectangular plastic food container. A second container was placed inside the first to create a sealed chamber. The chamber was sealed shut with paper clips and sterilised in an over at 80ºC for 24 h. Cotton seeds were surfaced sterilised in a 1% sodium hypochlorite and 5% ethanol solution for 5 min. After rinsing in sterile distilled water 10 seeds were placed in each or 20 chambers. To each chamber 10 mL of water was added and the 20 chambers were wrapped in plastic (see figure 2.2.1). The seeds were incubated for 2 d at 27ºC.

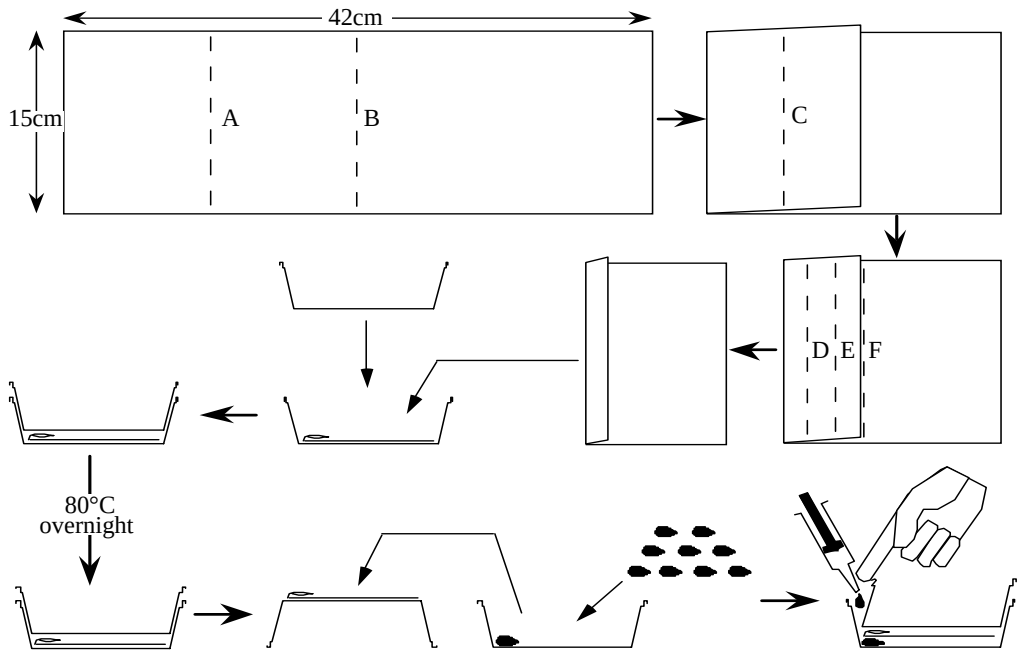

Figure 2.2.1 Preparation of chambers for germinating cotton seedlings (courtesy of David Nehl).

Inoculation of soil and planting of seedlings

The soil in each pot (700 mL milkshake cup as described above) was spread out in a plastic tray and mixed with 9 g of quartz sand (Sigma), then returned to the pot. For each of the three soils described above 15 pots were mixed with the *T. basicola*-sand inoculum (T) and 15 with sterile sand as a control (C), creating 6 treatments (R1-C, R1-T, 50/50-C, 50/50-T, NR1-C and NR1-T). A single seedling was transferred to each pot using sterile forceps and planted 2 cm below the surface (day 1). The plants were watered by placing the pots in approximately 5 cm of water and allowing the soil to absorb water to the top. The plants were grown on a 24 hour diurnal cycle; dark and 18ºC for 12 h then light and 25ºC for 12 h, until harvested. Watering was repeated on days 4, 6, 8, 11, 13, 15, 18, 20, 22, 25, 27 and 29.

Harvest

Due to seedling mortality three pots for each treatment were discarded. On day 15, half of the plants were harvested. The shoots were removed and weighed and placed in an oven at 60ºC for 24 hours then reweighed. The pots were soaked in 2g/L Calgon® (sodium hexametaphosphate) solution for 2 h. Roots were washed free from soil and examined for

disease. Roots were rated on a scale of 0 (no blackening of tap root) to 10 (complete blackening of tap root). The roots were placed in an oven at 60ºC for 24 hours then weighed.

On day 29 the remaining plants were harvested and measured as above except that the roots were also weighed before drying.

2.3 Testing the effect of *Trichocomaceae* in soil

Soil preparation and inoculation

Equal quantities of cultivated (R1) and non-cultivated (NR1) soil (as above) were mixed. The mixture was steamed at 65-75ºC for 3 h. Thirty pots (700 mL polystyrene cups) were filled with steamed soil. The soil in each pot was mixed with *T. basicola*-sand inoculum using the same method as the comparative soils experiment descried above.

Preparation of Trichocomaceae inoculum

Colonies of 35 isolates of fungi (see Appendix 1.) belonging to the family *Trichocomaceae* were grown on potato dextrose agar (PDA) for 7 d. A sterile solution of 0.02% Pulse™ (Nufarm Ltd., contains 1020 g/L polyether modified polysiloxane, a surfactant) was added to the surface of each colony. The colony surface was scraped with a sterile microscope slide and the resulting solution collected. The combined solution was passed through a 106 µm sieve to remove hyphal fragments and then centrifuged at 3000 rpm for 3 min. The supernatant was discarded and the spores resuspended in 0.02% Pulse™ solution made up to a total volume of 50 mL. A 1 mL aliquot was reserved for counting.

Planting and inoculation

Cotton seedlings germinated as above were grown for 2 d at 27ºC and then for 5 d at 16ºC. The seed husk was removed from the cotyledons using sterile forceps before planting. Seedlings were immersed in either a sterile 0.02% Pulse™ solution or the *Trichocomaceae* spore suspension (99.8 million spores per mL) before planting. A single seedling was planted in each pot with the cotyledons at the soil surface. The pots were

water (as above) on days 1, 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27 and 29. The plants were grown on a 24 hour diurnal cycle; dark and 18ºC for 12 h then light and 25ºC for 12 h, until harvested.

Harvest

Harvesting of plants was conducted as for the comparative soil experiment described above. Five plants were harvested on days 15, 22 and 29.

2.4 Secondary metabolite screening

Eight selected fungi (NR-1/1, NR-1/2, NR-1/6, R-1/1, OG-B/3, OG-B/5, CB/3 &CB/9 (see Appendix 1. for details) were grown separately for 7 d in 100 mL of nutrient broth (Amyl media, AM131), containing per litre 5 g bacteriological peptone, 5 g NaCl, 2 g yeast extract and 1 g beef extract. Each culture solution was filtered and the filtrate collected. The fungal matter was retained in the filter paper, dried and weighed. For each species an aliquot of filtrate equivalent to 50 mg dry weight of fungal biomass was passed through a 0.22 µm Millipore filter to sterilise. The sterile filtrate was added to 65 mL of sterile, molten PDA (45.08 g/L) at 50ºC and the total volume made up to 75 mL with sterile nutrient broth (formula as above). The solution was then poured equally into five 85 mm Petri dishes and left to set.

Each plate was inoculated with *T. basicola* by placing three 1 mm cubes of agar, excised from the edge of 7 d old *T. basicola* colonies, on the agar surface. The plates were then incubated at 21ºC in the dark. Colony radii were measured after 5, 7 and 10 d.

3 RESULTS

3.1 Fungi isolated

Altogether, 44 cultures of Trichocomaceae-like fungi were prepared; 42 isolates from seven soil samples collected around the Narrabri district and two isolates from foliar tissue of cotton plants. It is not known how many species were represented (see Appendix 1 for details).

3.2 Comparative soils experiment

No significant differences in plant weight were observed between soils for either the *T. basicola*-inoculated or the un-inoculated plants after 15 days. The un-inoculated plants showed no disease symptoms, whilst the inoculated plants had measurable disease symptoms (Table 3.2.1a).

Table 3.2.1a Plant weight and disease severity after 15 days growth. Letters indicate significant differences ($P < 0.05$).

Soil type and treatment	Shoot weight (g)		Root weight (g)		Disease index (0-10)
	Fresh	Dry	Fresh	Dry	
Cropped	0.63a	0.09a	-	0.04a	0.00a
Cropped + *Tb*	0.68a	0.08a	-	0.03a	4.83b
Mixed	0.69a	0.10a	-	0.04a	0.00a
Mixed + *Tb*	0.87a	0.10a	-	0.04a	4.17b
Uncropped	0.75a	0.09a	-	0.04a	0.00a
Uncropped + *Tb*	0.71a	0.08a	-	0.03a	2.50b

Disease severity was significantly lower in the uncropped soil and the mixed soil than in the cropped soil after 29 days. Disease severity in the uncropped soil was also significantly lower than in the mixed soil. The fresh shoot weight of plants grown in the cropped soil was significantly lower than plants grown in the uncropped soil and the mixed soil. Other significant differences in weight were observed but no pattern emerged (Table 3.2.1b).

Table 3.2.1b Plant weight and disease severity after 29 days growth. Letters indicate significant differences ($P < 0.05$).

Soil type and treatment	Shoot weight (g)		Root weight (g)		Disease index (0-10)
	Fresh	Dry	Fresh	Dry	
Cropped	1.46a	0.31ab	1.14ab	0.12ab	0.00a
Cropped + *Tb*	1.32a	0.28a	0.77a	0.11a	9.00b
Mixed	2.16bc	0.40b	2.11c	0.17c	0.00a
Mixed + *Tb*	1.91b	0.34ab	1.44bd	0.14ac	5.17c
Uncropped	2.98c	0.48b	2.14cd	0.17bc	0.00a
Uncropped = *Tb*	2.13bc	0.34ab	1.58cd	0.15ac	3.17d

3.3 Effects of reintroduced Trichocomaceae on *T. basicola*

No significant differences in plant size or disease severity were observed between Trichocomaceae-inoculated plants and control plants after growth for 15, 22 or 29 d under standardised conditions (Tables 3.3.1a-c).

Table 3.3.1a Plant weight and disease severity after 15 days growth.

	Shoot weight (g)		Root weight (g)		Disease index (0-10)
	Fresh	Dry	Fresh	Dry	
Control	1.09	0.15	0.85	0.07	8.20
With Trichocomaceae	1.01	0.13	0.80	0.07	5.80

Table 3.3.1b Plant weight and disease severity after 22 days growth.

	Shoot weight (g)		Root weight (g)		Disease index (0-10)
	Fresh	Dry	Fresh	Dry	
Control	2.24	0.39	1.60	0.14	8.40
With Trichocomaceae	1.47	0.26	1.23	0.11	9.40

Table 3.3.1c Plant weight and disease severity after 29 days growth.

	Shoot weight (g)		Root weight (g)		Disease index (0-10)
	Fresh	Dry	Fresh	Dry	
Control	2.85	0.66	1.88	0.22	5.20
With Trichocomaceae	2.22	0.55	1.50	0.19	6.20

3.4 The effects of secondary metabolites on *T. basicola*

The diameter of *T. basicola* colonies was 17% smaller than the control when grown on media amended with filtrate from the growth solution of one Trichocomaceae isolate, R-

1/1 (Figures 3.4.1 and 3.4.2). The diameters of *T. basicola* colonies were 3% larger than the control when grown on media amended with filtrate from the growth solutions of two Trichocomaceae isolates. There was no difference between the control and five of the eight growth-solution-filtrate-amended media (Figure 3.4.1).

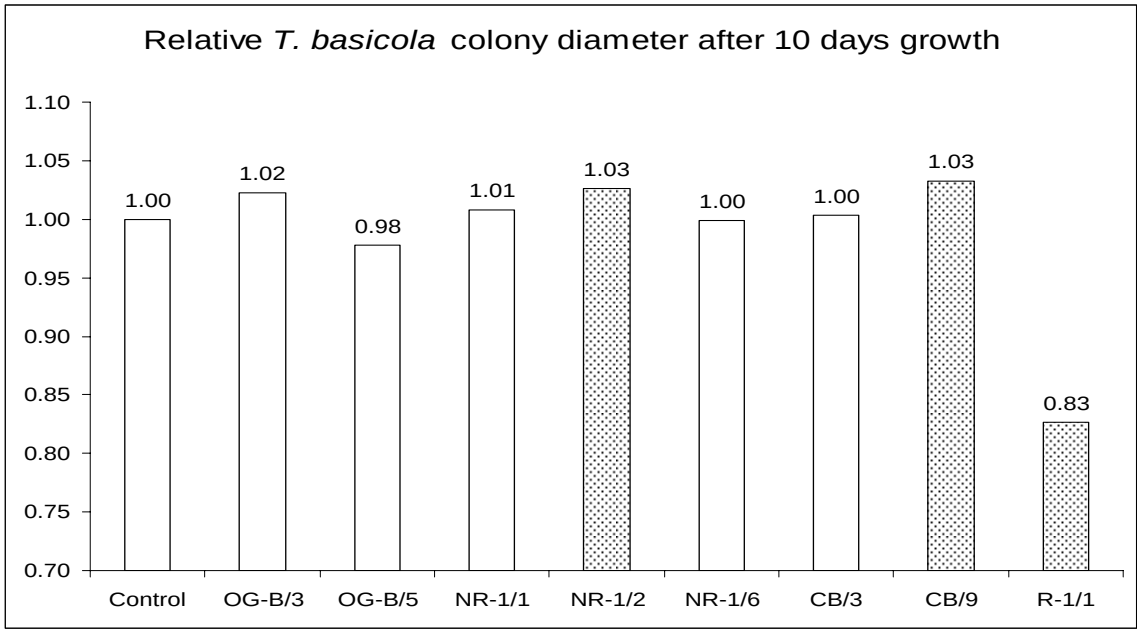

Figure 3.4.1 The diameter of *T. basicola* colonies relative to a control when grown on media amended with growth solution of eight saprotrophic soil fungi.

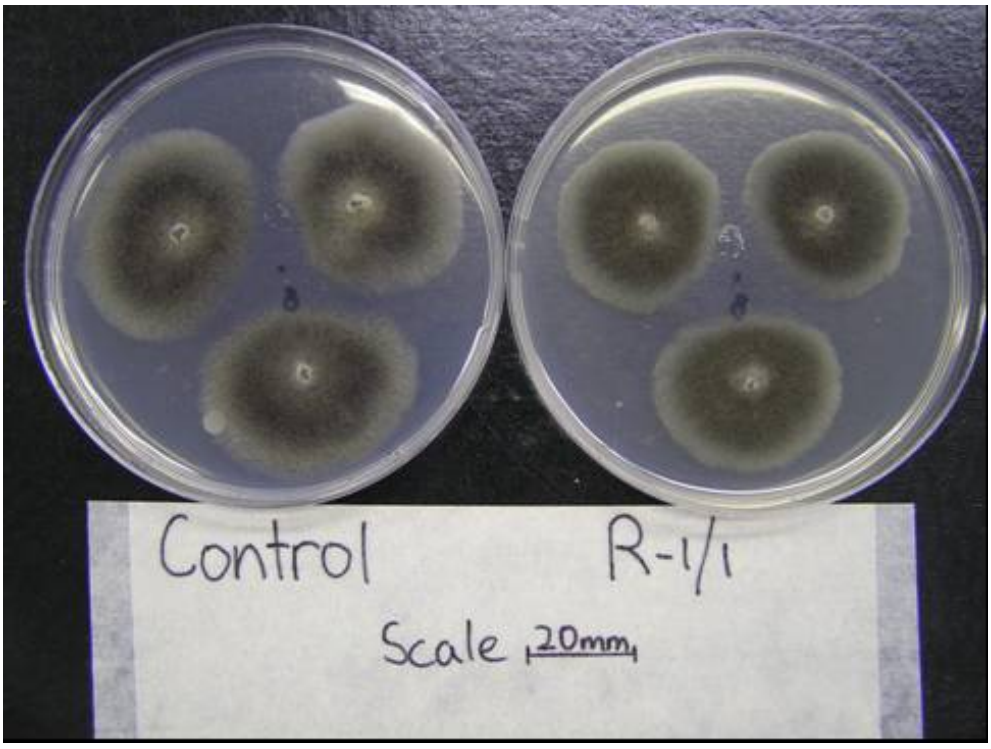

Figure 3.4.2 Photograph of *T. basicola* when grown on media amended with growth solution from culture of soil saprotroph (R-1/1) or grown on unamended media (control).

4 DISCUSSION

Good management of soil biodiversity is essential for efficient and productive farming. Manipulation of soil biodiversity may provide a means for managing soil borne pathogens in crop environments. Experiments described here failed to show that manipulation of microbial diversity in crop soil has a significant impact on black root rot in cotton plants.

Comparison of disease severity between cropped and uncropped soils showed that cotton plants grown in uncropped soils recovered faster from black root rot (figures 3.2.1a-b). It isn't clear whether this was due to biological factors or abiotic factors. Plants grown in cropped soil were significantly smaller after 29 days than those grown in uncropped soil (figure 3.2.1b), although the size difference was significant only for shoot fresh weights and only apparent for other measurements, suggesting that faster recovery from black root rot allowed the plants to grow faster. However this pattern was mirrored when plants were not inoculated with *T. basicola*. Although the inoculated plants had less disease when grown in uncropped soil, all plants were larger when grown in uncropped soil indicating that the lower disease rate is more likely to be because of more rapid progression through the disease cycle. *T. basicola* infects the outer epidermis of cotton seedlings after about a week's growth and causes significant blackening. After about five to six weeks, cotton plants in the field slough of the outer epidermal layer and recover from disease symptoms (Nehl, personal communication). The results of the soils comparison are inconclusive but suggest that there may be a relationship between soil biodiversity and disease severity. In order to fully understand the situation it is necessary to compare a larger number of soils. Also, whilst there is evidence that cropped soils are less diverse than uncropped or natural soils, it would be better to measure the biodiversity of all soils compared.

Reintroduction of selected fungi did not affect disease severity in cotton seedlings (Figures 3.3.1a-c). This could be due to a number of factors: the selected microbes do not possess the ability to antagonise *T. basicola*; the inoculation method failed to set up suitable conditions for antagonism; or the density of *T. basicola* spores was sufficient to

overcome the antagonistic action of the reintroduced microbes. Firstly, it has been shown that some soil saprotrophs have antagonistic action against *T. basicola* (Tashlieva, 1980) hence the limited range of microbes reintroduced may not be a representative sample. Secondly, an ecosystem is a complex and dynamically functioning entity which is not represented adequately by reintroduction of microbes into controlled conditions. Finally, *T. basicola* was reintroduced at a density much higher than observed field-levels; inoculation density may have overloaded the antagonistic potential of the artificial system. These considerations indicate that ecosystem complexity makes investigation of the impact of biodiversity on crop pathogenesis difficult.

Screening of eight soil saprophytes isolated from the Namoi Valley revealed that at least one fungus produces metabolites that are inhibitory to *T, basicola*. Although only a 17% inhibition of radial growth was observed it is possible that wider screening of microbes native to the region would reveal many more inhibitory microbes. It is possible that a microbe that produced strongly inhibitory metabolites could be found and utilised in management of the disease. Alternatively it is possible that a large number of weakly inhibitory microbes already exist in native soils, and that promoting these microbes in crop soils may help in the management of the pathogen.

The three experiments reported here indicate that there may be some truth to the idea that greater soil biodiversity could be useful in the management of crop pathogens.

5 ACKNOWLEDGEMENTS

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7 APPENDIX 1. List of cultures with collection details.

Number	Location details	GPS-lat	GPS-long
CB/1	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/2	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/3	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/4	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/5	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/6	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/7	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/8	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/9	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
CB/10	Bulyeroi Rd, near Collins Bridge Namoi R. Under Eucalyptus microtheca	30º11.444'S	149º30.021'E
JC/1	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/2	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/3	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/4	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/5	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/6	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/7	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/8	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/9	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E
JC/10	Ten mile Dam, Jack's creek state forest. Under Eucalyptus sp and Callitrus glauca	30º27.557'S	149º43.539'E

Number	Location details	GPS-lat	GPS-long
NR-1/1	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
NR-1/2	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
NR-1/3	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
NR-1/4	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
NR-1/5	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
NR-1/6	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
NR-1/7	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
NR-1/8	ACRI, near field R1, stock reserve.	30º12.714'S	149º34.999'E
R-1/1	ACRI, field R1, row 15 from river, 25m from tail drain.	30º12.705'S	149º35.030'E
R-1/2	ACRI, field R1, row 15 from river, 25m from tail drain.	30º12.705'S	149º35.030'E
O-2/1	ACRI, field old 2, Thielaviopsis basicola infested, 2nd year wheat following continuous cotton. Row 28 from west, 15 meters from tail drain.	30º12.296'S	149º35.580'E
O-2/2	ACRI, field old 2, Thielaviopsis basicola infested, 2nd year wheat following continuous cotton. Row 28 from west, 15 meters from tail drain.	30º12.296'S	149º35.580'E
O-2/4	ACRI, field old 2, Thielaviopsis basicola infested, 2nd year wheat following continuous cotton. Row 28 from west, 15 meters from tail drain.	30º12.296'S	149º35.580'E
O-2/5	ACRI, field old 2, Thielaviopsis basicola infested, 2nd year wheat following continuous cotton. Row 28 from west, 15 meters from tail drain.	30º12.296'S	149º35.580'E
OG-A/2	Orange grove farm, Eulah creek Rd. Under Cadellia pentastylis and Callitrus glauca.	30º20.627'S	150º00.566'E
OG-A/4	Orange grove farm, Eulah creek Rd. Under Cadellia pentastylis and Callitrus glauca.	30º20.627'S	150º00.566'E
OG-A/5	Orange grove farm, Eulah creek Rd. Under Cadellia pentastylis and Callitrus glauca.	30º20.627'S	150º00.566'E
OG-B/1	Orange grove farm, Eulah creek Rd. Near base of Gossypium sturtuanum var. nandewarens.	30º20'633'S	150º00.758'E
OG-B/2	Orange grove farm, Eulah creek Rd. Near base of Gossypium sturtuanum var. nandewarens.	30º20'633'S	150º00.758'E
OG-B/3	Orange grove farm, Eulah creek Rd. Near base of Gossypium sturtuanum var. nandewarens.	30º20'633'S	150º00.758'E
OG-B/4	Orange grove farm, Eulah creek Rd. Near base of Gossypium sturtuanum var. nandewarens.	30º20'633'S	150º00.758'E
OG-B/5	Orange grove farm, Eulah creek Rd. Near base of Gossypium sturtuanum var. nandewarens.	30º20'633'S	150º00.758'E
DB-L	From cotton basal leaf, ACRI field old 2.	30º12.296'S	149º35.580'E
B-AL	From cotton apical leaf, ACRI field old 2.	30º12.296'S	149º35.580'E