

Final Report

On Farm Series | Cotton Research & Development Corporation

Part 1 - Summary Details

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

CRDC Project Number: CSP156C

Project Title: The potential for native *Fusarium* to give rise to new cotton field pathogens

Project Commencement Date: 1 Jan, 2004 **Project Completion Date:** 30 Jun, 2007

CRDC Program: 3 Crop Protection

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Part 3 – Final Report Guide (due 31 October 2007)

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

Background

1. Outline the background to the project.

Fusarium wilt, caused by the fungal pathogen *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*), is a destructive disease of cotton worldwide. In Australia, the disease was first reported in 1993 in Brookstead/Cecil Plains, QLD and Boggabilla, NSW. It is now widespread in most cotton growing regions, causing significant losses. Work in project CSP113C clearly demonstrated that the *Fov* strains responsible for the disease in Australia are genetically related to native *Fusarium oxysporum*. This has significant implications for further development of *Fov* and therefore for the industry.

In project CSP113C, pathogenic *Fusarium oxysporum* was consistently found in rhizosphere of wild cotton populations. Isolates were capable of inducing mild, but typical Fusarium wilt symptoms on susceptible cotton plants (Siokra 1-4). Based on the fingerprints generated using molecular markers (AFLPs), these pathogenic *Fusarium oxysporum* isolates can group into 5 distinct lineages (designated A, B, C, D, and E). Although some isolates from all the 5 lineages are able to cause mild Fusarium wilt symptoms, the incidence of pathogenicity is significantly higher in lineage A.

The 2 *Fov* strains devastating the Australian cotton fields (VCGs 11 & 12) are members of lineage A, a lineage that is genetically distinct from the overseas *Fov* strains. This suggests that the appearance of Fusarium wilt of cotton in Australia has resulted from recent evolutionary change in pathogenicity of the native, weakly pathogenic *Fusarium oxysporum* types occurring in lineage A. This process has most likely been facilitated by continuous, wide-scale cropping of susceptible cotton cultivars, as appears to be the case for the evolution of *Fusarium oxysporum* f. sp. *melonis* attacking melons (Katan *et al.*, 1994). Consequently, it is essential to know more about the genetic structure of *Fusarium oxysporum* occurring in the soil of cotton fields and the evolutionary processes that promote the development and spread of highly virulent forms of *Fusarium oxysporum*.

At present very little is known about the genetic diversity of *Fusarium oxysporum* in the soil of cotton fields. Lineages A and B have been detected in native soil adjacent to cotton fields, suggesting that they are not exclusively associated with native cottons, but their existence and prevalence in the soil of cotton fields is unknown. If mildly pathogenic forms of *Fusarium oxysporum* are also common in cotton fields, this would substantially increase the likelihood that further new pathotypes of *Fov* may emerge in the future.

Breeding for resistance is the most effective management strategy for *Fov* control. However, the resistances to be selected are determined by the *Fov* genotypes used during the screening process. The evolution of novel *Fov* forms from lineage A or other wild lineages significantly changes the breeding environment. A better understanding of the processes conditioning the evolution of pathogenicity in *Fusarium oxysporum* is of great importance to risk assessment of novel *Fov* forms and durability of resistant cotton cultivars. The two main events during this process are spontaneous mutation which generates new genotypes at very low rates and directional selection that then favours the emergence of isolates capable of overcoming the current resistance barriers. Recent work has demonstrated that the selection coefficients of plants for fungal pathogens (the rate of change in frequency of an allele) range from 0.025 to 0.622, suggesting that

selection plays a very important role in the appearance of novel *Fov* pathotypes (McDonald & Linde 2002). Deploying cultivars resistant to VCGs 11 and 12, but susceptible to novel *Fov* pathotypes could provide intense selection pressure for increasing virulence.

Early knowledge of the genetic diversity of potential *Fov* pathotypes, the processes by which virulence develops, and the probability that new pathogens can spread is essential to long-term efforts to control the disease in Australia. This information will have a significant impact on disease resistance breeding strategies as well as encouraging the deployment of resistant cotton cultivars throughout the industry – not just in *Fov* infected fields. In addition, early detection of new *Fov* genotypes is also critical for containment and sanitation protocols.

References: Katan *et al.* (1994). *Phytopathology* **84**: 153–157.
McDonald & Linde (2002). *Euphytica* **124**: 163–180.

Objectives

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

Fusarium oxysporum genotypes occurring in wild cotton populations could extensively expand the genetic diversity of cotton-adapted genotypes of *Fov*, thereby greatly widening the pathogen base against which breeders have to select and potentially setting back the gains in resistance that have already been made. Understanding the evolutionary processes involved in these changes is critical. To best position breeders to cope with this possibility and to ensure that ongoing breeding efforts, i.e., resistances from wild cottons, biotechnological solutions, and selective breeding, are successful, it is essential to determine (A) the incidence and genetic diversity of native *Fusarium oxysporum* in the soil of cotton fields, (B) the evolutionary process of native *Fusarium oxysporum* lineages to become virulent pathotypes of *Fov*, and (C) the potential for novel *Fov* pathotypes to compete with existing VCGs 11 and 12.

- A. Assessing the risk of novel *Fov* genotypes emerging from native *Fusarium oxysporum*

A detailed survey of soils and diseased plants from 14 farms (2 in Narrabri, 3 in Moree, 4 in Boggabilla, and 5 in the Darling Downs) was conducted. A total of 180 soil samples and 240 diseased plants were collected, from which 1439 isolates were obtained including 1199 *Fusarium oxysporum* isolates from soils and 240 *Fov* isolates from plant samples. The genetic diversity of *Fusarium oxysporum* resident in cotton field soils and *Fov* currently causing the disease in fields was determined. All isolates were genotyped using AFLPs established under projects CSP113C and CSP155C and grouped into lineages. Twelve lineage representatives were sequenced for EF-1 α and mtSSU genes. In addition, 234 lineage A isolates of *Fusarium oxysporum* from soils were tested for pathogenicity on Siokra 1-4.

- B. Investigating the evolutionary processes involved in wild *Fov* becoming virulent

Fifty wild *Fov* isolates (15 in lineage A, 17 in lineage B, and 18 in lineage E), recovered from rhizosphere soils of native *Gossypium* populations under project CSP113C, were tested for their potential to become virulent pathogens subject to continuous exposure to susceptible cotton plants. Ten inoculation – re-isolation – re-inoculation cycles were completed, which generated 1000+ offspring isolates including 650 from a lineage A isolate (2613). All these offspring isolates were genotyped using AFLPs. Significant increase in virulence was seen in the lines from isolate 2613. The extent to which virulence of isolate 2613 has increased was

determined by comparing the disease caused by 11 offspring isolates generated in different infection cycles and 2 VCG 11 reference isolates.

C. Assessing the competitive abilities of *Fov* genotypes

A total of 157 *Fov* isolates were obtained from diseased plants collected from a Boggabilla field in 3 successive cotton growing years (66 in 2002, 25 in 2004, and 66 in 2006). Isolates were genotyped using AFLPs and frequencies of *Fov* genotypes in isolates were determined.

In the glasshouse, 100+ two-week-old seedlings of 2 cotton cultivars, Siokra 1-4 and Sicot 189, were inoculated with a 50:50 spore mixture of genotype A, the dominant form of *Fov* in 2002, and genotype B, the dominant form of *Fov* in 2006, using the root dipping method. A total of 500 cultures were recovered by isolating 10 single spores from each of 50 randomly chosen diseased plants and genotyped using AFLPs. The incidence of genotypes A and B in sampled diseased plants were determined.

Competitive abilities of VCGs 11 and 12 were determined using the same methodology. About 850 single-spore cultures were genotyped using the AFLPs.

D. Collecting *Fov* isolates from areas that have not been surveyed in the past

A total of 995 *Fov* isolates were collected from 23 cotton fields in Theodore, St George, Bourke, Warren, and Mungindi. These fields have never been sampled in previous surveys.

E. Determining the origin of the Mungindi *Fov* strain

A novel *Fov* strain was found in Mungindi in 2005. The genetic relationships between the Mungindi strain and overseas *Fov* races, VCGs 11 and 12 were determined using the AFLPs.

F. Determining the origin of the Mungindi *Fov* strain

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Methods

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

A. To assess the risk of novel *Fov* pathotypes emerging in cotton fields, *Fusarium oxysporum* was isolated from disease-infested and disease-free soil samples and *Fov* was recovered from diseased plant samples. Isolates were genotyped using the AFLPs and identified to VCGs or lineages based on the genetic similarity to the reference isolates established under projects CSP113C and CSP155C. All non-VCG 11 and 12 isolates in lineage A were tested on Siokra 1-4 for pathogenicity.

Several methods and media were tested in a preliminary trial for efficacy in isolation of *Fusarium oxysporum* from soil samples. The best result was observed when approximately 0.5 g of soil was sprinkled onto Peptone-PCNB agar plates. It was a lot easier to distinguish *Fusarium oxysporum* colonies from other soil fungi. This protocol was successful in isolation of *Fusarium oxysporum* from all 180 soil

samples collected in this project. Isolation of *Fov* from diseased plant samples was conducted on 25% potato dextrose agar containing 0.05% streptomycin sulphate, on which *Fov* can grow well, while the growth of most bacteria contaminants is well inhibited.

- B. To investigate the evolution of virulence, weakly pathogenic isolates of *Fusarium oxysporum* collected from wild cotton soils were exposed to directional selection for pathogenicity by repeated inoculation – re-isolation – re-inoculation (10 infection cycles or generations) on susceptible cotton cultivar Siokra 1-4. Variation in virulence was determined by comparing the disease severity observed at different generations. Lines showing significant increases in virulence were genotyped using the AFLPs to determine if genetic changes accompany the virulence changes and how they could be used in the future to identify the genes responsible for the switch.

Significant increases of virulence were observed, suggesting that directional selection for pathogenicity was successfully achieved in multiple generations of repeated inoculation – re-isolation – re-inoculation. Although genotyping all offspring isolates before they entered the next generation was time-consuming (at least 2.5 weeks), it was critically important since this ensured that no contaminants would be carried into next generations. On the other hand, only 30 plants were used for each isolate in a generation due to the limited glasshouse space and 10 generations were completed in this work due to the relatively shorter length of the project (3 years), the extent to which virulence has increased could be more significant if more plants were used in each generation and more infection cycles could be conducted.

- C. The competitive ability of *Fov* genotypes was determined by carrying out a field assessment and glasshouse experiments. For the field assessment, the frequencies of *Fov* genotypes in a Boggabilla field were assessed by collecting diseased plants in 3 successive cotton growing years, isolating *Fov*, genotyping isolates, and calculating the frequencies of different *Fov* genotypes in the total isolates. In the glasshouse experiments, seedlings of susceptible cotton cultivar Siokra 1-4 and tolerant cotton cultivar Sicot 189 were inoculated with a 50:50 spore mixture of 2 dominant genotypes. The incidence of the 2 genotypes on a total of 50 diseased plants were determined by isolating 10 single spores from each of diseased plants, genotyping isolates, and identifying the genotype responsible for infection on diseased plants. In addition, competition between VCGs 11 and 12 was also determined using the similar protocol except that inoculation was conducted using 3 methods and the trials were carried out on one cotton cultivar (Siokra 1-4).

Three inoculation methods – root dipping, soil drenching, and stem puncturing were used in the study of competition between VCGs 11 and 12. Consistent results were seen despite disease incidence caused by stem puncturing was significantly lower than that caused by root dipping, suggesting that the major infection sites of *Fov* is on the roots. In addition, disease incidence caused by soil drenching showed relatively higher level of variation, suggesting that infection is mediated or at least influenced somehow by other soil micro-organisms.

- D. *Fov* was recovered from diseased plants collected from farms that had never been sampled previously in Theodore, St George, Moree, Boggabilla, and Mundindi. Single-spored cultures were produced, and stock spores suspensions were stored in 15% glycerol at -80C.

Some diseased plants collected from these less investigated regions looked different from those found commonly in the Darling Downs, Boggabilla, and Moree,

suggesting that the disease in these newly sampled regions might be caused by novel *Fov* pathotypes although there was another possibility that such a difference is related to region-specific soil condition, climate, or agronomic practices.

- E. DNA was extracted from the Mungindi strain provided by Dr Linda Smith in QDPI. Its origin was determined by determining the genetic relationships to native *Fusarium oxysporum* and overseas *Fov* races using AFLPs.

No justification or discovery

Results

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

- A. Assessing the risk of novel *Fov* genotypes emerging from native *Fusarium oxysporum*

- 1) Genetic diversity of soil-borne *Fusarium oxysporum* in cotton growing regions

Results from 1069 soil-borne *Fusarium oxysporum* isolates collected from 20 cotton fields (7 *Fov*-free and 13 *Fov*-infested) and 4 uncultivated lands in the Narrabri, Moree, Boggabilla, and Cecil Plains regions showed that 3 lineages (A, B, and E) occur in these cotton-growing regions (Table 1). The presence of lineage A in cotton fields complicates the development of disease control strategies because novel *Fov* pathotypes could evolve from lineage A. In addition, it needs to be taken into account when molecular diagnostic tools are developed as non-pathogenic isolates of lineage A may give false positives given their close genetic relationships to *Fov*.

- 2) Difference in lineage composition of soil *Fusarium oxysporum* population between cotton fields and uncultivated land

Table 1. Summary of the numbers of total isolates and isolates in different lineages from 24 soil-borne *Fusarium oxysporum* populations from *Fov*-free fields, *Fov*-infested fields, and uncultivated land in cotton growing regions.

Pop.	Origin	# Isolates	A	B	E	Others
Fov-free field						
1	Narrabri	7	0	1	6	0
2	Narrabri	10	0	0	10	0
3	Narrabri	35	2	2	31	0
4	Narrabri	42	0	2	38	2
5	Boggabilla	45	0	1	44	0
6	Boggabilla	43	0	0	43	0
7	Darling Downs	35	5	1	29	0
		217	7	7	201	2
Fov-infested field						
8	Moree	44	0	0	44	0
9	Moree	45	1	0	44	0
10	Moree	53	0	5	48	0
11	Moree	54	0	0	54	0
12	Boggabilla	67	12	0	55	0
13	Boggabilla	60	15	3	41	1
14	Boggabilla	52	2	1	49	0
15	Boggabilla	55	10	2	43	0
16	Darling Downs	41	31	3	7	0
17	Darling Downs	52	29	2	20	1
18	Darling Downs	38	5	2	31	0
19	Darling Downs	31	13	2	16	0
20	Darling Downs	50	34	0	16	0
		642	152	20	468	2
Uncultivated land						
21	Boggabilla	62	6	14	42	0
22	Boggabilla	51	26	18	7	0
23	Boggabilla	48	2	30	15	1
24	Darling Downs	49	21	24	4	0
		210	55	86	68	1
	Total	1069	214	113	737	5

Lineage E is the dominant form of *Fusarium oxysporum* in cotton fields regardless of occurrence of the disease (Figure 1). Previous results from project CSP113C showed that lineage B is the most prevalent form of *Fusarium oxysporum* in wild cotton soils across the country, which is consistent with the observation from uncultivated land in the current project (Figure 1). The shift of dominant form of *Fusarium oxysporum* from lineage B to lineage E in cotton fields suggests that the fungal population is considerably influenced by cropping and cultivation practices, *i.e.* fertiliser and fungicide applications. However, it is

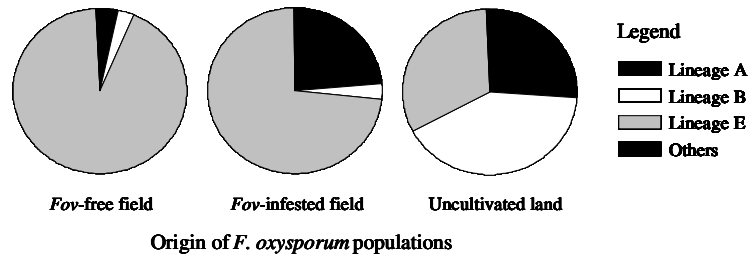


Figure 1. Incidence of Lineages A, B, and E of soil-borne *Fusarium oxysporum* in different soils

unclear how such changes in lineage composition of *Fusarium oxysporum* population are related to the occurrence of Fusarium wilt of cotton in the fields.

3) Genetic variation and population structure of *Fov*

Genetic variation among 348 *Fov* isolates collected from diseased cotton plants in 31 fields in 6 cotton growing regions in New South Wales and Queensland in 2002 and 2004 was analysed using AFLPs. Twenty-eight haplotypes were identified based on the 146 polymorphic bands generated with 4 *EcoRI*:*MseI* and 4 *HindIII*:*MseI* primer pairs. The 28 haplotypes separated into 2 groups with 21 in Group I (VCG 11) and 7 in Group II (VCG 12). Group I was widespread, occurring in all sampled regions and all but one of the sampled fields, while

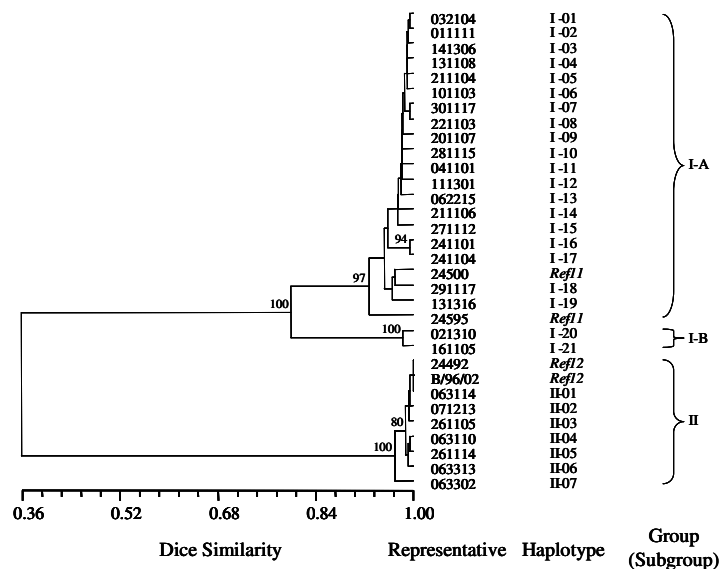


Figure 2. Dendrogram based on the Dice coefficient constructed by UPGMA cluster analysis of 146 polymorphic bands from 28 haplotype representatives generated with 8 AFLP primer pairs.

Group II was restricted to 3 fields in the Boggabilla region. Group I was further divided into 2 subgroups, and Subgroup I-B represents a new VCG since isolates in this subgroup are incompatible to either of the 2 known VCGs (Figure 2). A

Table 2. Analysis of molecular variance (AMOVA) for populations of *Fov* from (A): 31 fields across six regions; and (B) nine fields in the Boggabilla region.

Analysis	Source of variation	d.f.	Sum of squares	Variance components	% of the total variance	P-value ^a
A	Among regions	5	478.3	0.3391	3.9	0.4868
	Within regions	25	1999.4	6.9793	79.8	<0.0001
	Within fields	317	252.2	1.4267	16.3	
	Total	347	2929.9	8.7451		
B	Among fields	8	1983.5	18.6635	86.3	<0.0001
	Within fields	111	330.0	2.9730	13.7	
	Total	119	2313.5	21.6365		

preliminary assay of virulence among representatives of these haplotypes suggests that the genetic differentiation is accompanied by changes in virulence. No clear population structure was observed at the national level with only 3.9% of the total genetic variation attributable to differences among regions. When each region was analysed separately, clear structure was observed in the Boggabilla region with 86.3% of the total genetic variation due to differences among fields (Table 2).

B. Investigating the evolutionary processes involved in wild *Fov* becoming virulent

1) Repeated inoculation – re-isolation – re-inoculation cycles in the glasshouse

Of the 50 ancestral strains of native *Fusarium oxysporum* from wild cotton soils that were passed through cotton in this study, only 8 (7 lineage A and 1 lineage E) strains caused foliar symptoms in the first infection cycle and were passed to the second cycle. Among these 8 strains, 7 were isolated from soil collected from around *G. sturtianum* populations in Northern Territory and South Australia. Only one strain, 2613, completed 10 infection cycles on cotton, while all the others lost the ability to cause detectable foliar symptoms at various stages during the serial passage process. Nevertheless, variation in virulence was observed in the strain 2613 lineage and the others which failed to complete all serial passages, although some of that variation was probably caused by differences in environmental conditions under which the trials were conducted.

In addition, 47 offspring isolates were obtained from strain 2613 by conducting similar serial passage trials on water agar.

2) Genetic diversity of offspring isolates

A total of 136 isolates were genotyped, including the ancestral strain, 2613, and a sub-sample of its offspring isolates obtained from different infection cycles (88 isolates) and water agar (47 isolates). Forty-six unambiguous polymorphic markers were identified due to their absence in at least one isolate. Of these markers, 12 were observed in offspring isolates from both cotton and agar; 33 markers were found only in isolates generated on cotton, and 1 marker was found only in isolates generated on water agar.

Compared with the fingerprints of the ancestral strain, 16 haplotypes representing 22 mutants were identified among the 135 offspring isolates genotyped in this study (Figure 3). Fourteen mutant haplotypes were identified

among the 88 offspring isolates passed through cotton, while only 3 mutant haplotypes were seen among the 47 offspring isolates from water agar. Only one haplotype (C6-i and A5-e) was detected among the offspring isolates of both sources (Figure 3). There were no significant phylogenetic relationships between these mutant offspring isolates. For example, 2 mutant isolates generated from the 9th passage assay on cotton (C9-a and C9-f) occur in the same haplotype (Figure 3), but their ancestors (C1-a and C1-b) have already been separated since the 2nd assay. This reflects the lack of association between virulence traits and the particular AFLP markers assessed. A similar lack of association has been shown in many other fungal pathogens.

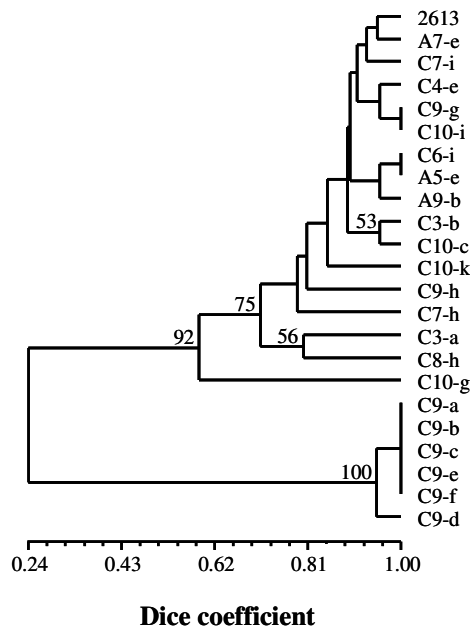


Figure 3. Dendrogram based on the Dice coefficient constructed by UPGMA cluster analysis of 46 polymorphic fluorescent AFLP markers from the ancestral strain 2613 and 22 mutant offspring isolates generated with four fluorescent AFLP primer pairs. The sources of offspring isolates are labelled by C (cotton) and A (agar) in front of isolate codes.

- 3) Increase in virulence of weakly pathogenic *Fusarium oxysporum* Two final comprehensive comparison trials were conducted. In trial I which involved 19 offspring isolates including one lineage (C1-b to C10-i) generated from 10 infection cycles on cotton and one lineage (A1-b to A9-j) generated from 9 passage assays on agar, no significant difference in virulence was observed in offspring isolates generated from the first 3 passage assays regardless of source. However, from the 4th assay on, offspring isolates from cotton showed higher level of virulence than those from agar. A significant difference in virulence was found between offspring isolates of different sources from the 9th assay, while the offspring isolate from the 10th assay on cotton (C10-i) was even more virulent than those from previous assays (Figure 4).

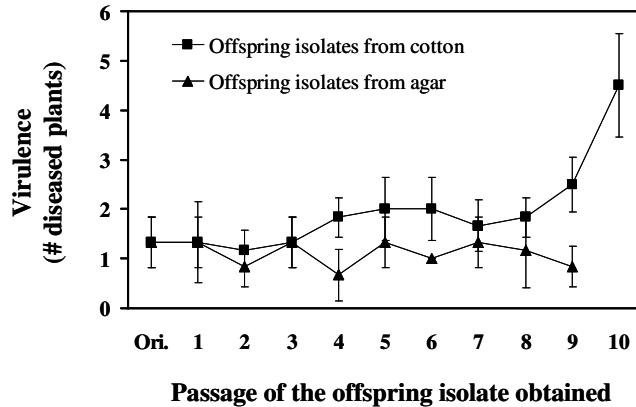


Figure 4. Variation of virulence in 2 lines of offspring isolates of the ancestral strain 2613 generated from serial passages on cotton and agar. Virulence is represented by the number of plants that became diseased out of 15 inoculated plants. The values are mean of 6 replicates from 2 independent trials. The bars are standard deviations.

In trial II which compared the virulence of 12 offspring isolates obtained from the 3rd, 7th, and 10th infection cycles respectively, when these isolates were grouped based on infection cycles, no significant difference was observed between the ancestral strain and any of the 3 groups. However, when individual isolates were compared, the virulence of isolates C10-h and C10-i (3.5 and 2.7) was significantly higher than the virulence of both the ancestral strain (1.6) and other offspring isolates used in the trial (ranging from 0.1 to 1.8) ($P < 0.01$; L.S.D._{0.01} = 0.93) (Figure 5) This study provides empirical evidence that the evolution of virulence in *Fov* occurs in a continuous process. As offspring isolates from the most diseased plants were serially passed on susceptible cotton plants, virulence was observed to gradually increase in some lineages, suggesting the successive accumulation of mutations for increased virulence.

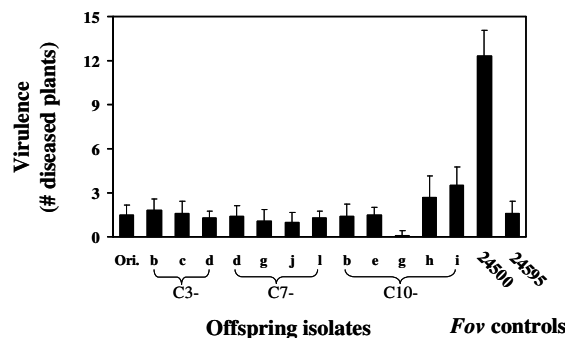


Figure 5. Virulence of offspring isolates of the ancestral strain 2613 generated from the 3rd, 7th, and 10th serial passage on cotton. Virulence is represented by the number of plants that became diseased out of 15 inoculated plants. The values are mean of 10 replicates from 2 independent trials. Each column represents an isolate, and the bars, standard deviations. The last two columns are reference field *Fov* isolates.

C. Assessing the competitive abilities of *Fov* genotypes

1) Temporal variation of *Fov* in the field

Temporal variation of *Fov* population was observed in a Boggabilla field (Figure 6). 157 isolates were recovered from diseased plants in 3 successive cotton growing seasons; among these 4 AFLP genotypes were detected. In 2002, the population was dominated by genotype 11-A, that accounted for 89% of the isolates, but genotype 11-B occurred at a frequency of only 2%. In 2004, the frequency of 11-A declined to 36%, while that of 11-B increased to 48%. This trend continued such that in 2006, genotype 11-B dominated the population, accounting for 77% of isolates, while genotype 11-A was undetectable.

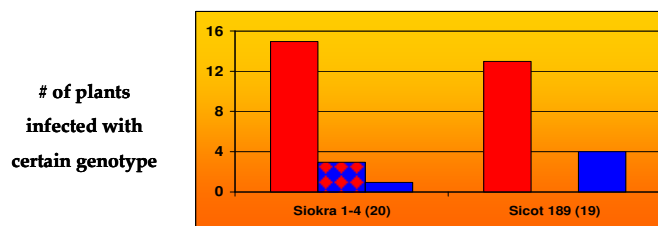


Figure 7. Number of plants infected by genotype 11-A ■, genotype 11-B ■, or both ■ on 2 cotton cultivars

2) Competitive interactions between *Fov* genotypes of VCG 11 in the glasshouse

Virulence of genotypes 11-A and 11-B was compared on 2 cotton cultivars (Siokra 1-4 and Sicut 189). No significant difference was found between the 2 genotypes, suggesting that the build-up of genotype 11-B in the field was unlikely to be the result of variation in virulence. On the other hand, competition was observed when interaction between the 2 *Fov* genotypes was assessed experimentally. When plants were inoculated with a 50:50 spore mixture of the 2 genotypes, disease was caused by infection of a single genotype on 17 of the 20 Siokra 1-4 plants and all Sicut 189 plants, suggesting that infection of a plant by one genotype can exclude infections by others. In addition, the 2 pathogen genotypes had different frequencies of occurrence on the 2 cotton types. Genotype 11-A had a lower incidence on Sicut 189 while genotype 11-B showed the reverse

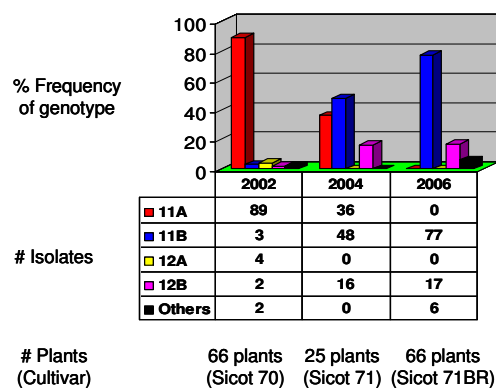


Figure 6. Number and frequency of *Fov* genotypes in isolates collected from a Boggabilla field in 3 successive cotton growing years

(Figure 7). This appears to be consistent with the correlation found between the increasing incidence of genotype 11-B and use of more resistant cotton cultivars in the field, suggesting that cotton plays an important role in temporal variation of *Fov* populations.

3) Competitive interactions between VCGs 11 and 12 in the glasshouse

The same methodology was used to determine interaction between VCGs 11 and 12 on cotton cultivar Siokra 1-4 except that plants were inoculated with a 9:1 or 1:9 spore mixture of the 2 VCGs using 3 methods. Similarly, competition was observed between the 2 VCGs. When the root-dip method was used, all 10 inoculated plants were infected by VCG 11 when the inoculum contained 9 parts of VCG 11 and 1 part of VCG 12. Moreover, 9 out of 10 inoculated plants were also infected by VCG 11 when the inoculum contained 1 part of VCG 11 and 9 parts of VCG 12 (Figure 8). This suggests that VCG 11 is more aggressive than VCG 12. Similar results were also obtained when the soil drenching and stem injection methods were used. On the other hand, dual infection, *i.e.*, both VCGs were recovered from a diseased plant, was not commonly found on the plants that were even inoculated using the stem injection method, implying that competitions are perhaps about both infection

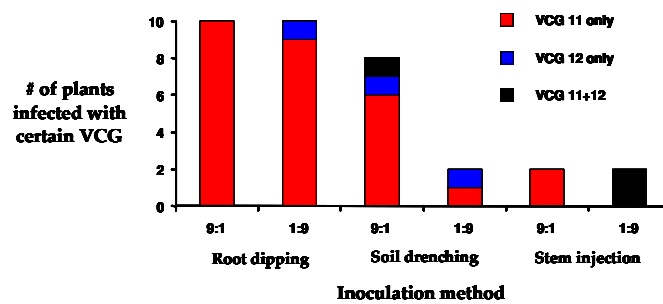


Figure 8. Comparisons in numbers of diseased plants infected by VCG 11, VCG 12, or both when different inocula and inoculation methods were used

process and performance of different *Fov* genotypes within plants.

D. Collecting *Fov* isolates from areas that have not been surveyed in the past

1) Geographic variation in prevalence of soil *Fov* among regions

Fusarium oxysporum occurs at similar levels of prevalence in the soil of *Fov*-infested fields in the Darling Downs, Boggabilla, and Moree regions. However, the frequency of *Fov* in *Fusarium oxysporum* population significantly declines from a mean of 23% in the Darling Downs soil to below 2% in Moree soil (Figure 9). One possibility is that shifts in saprophytic ability may be responsible for the significant differences in prevalence of *Fov* (primarily represented by VCG 11 from the Darling Downs) between the Darling Downs and Moree. Given the lack of differences in disease incidence and severity between the 2 regions, this

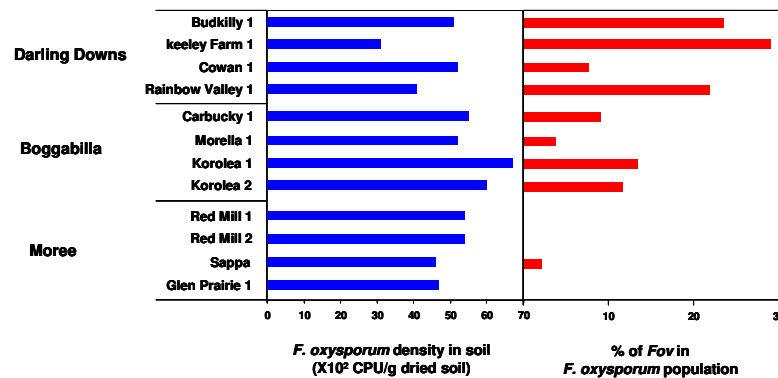


Figure 9. Prevalence of *Fusarium oxysporum* and frequency of *Fov* in *Fusarium oxysporum* in soils collected from diseased fields in different regions.

suggests that *Fov* aggressiveness may have increased in Moree relative to the Darling Downs since *Fov* genotypes are likely to differ in saprophytic ability to cope with other micro-organisms and various soils. This also suggests the possibility of a trade-off that has occurred between saprophytic ability and aggressiveness in *Fov*.

E. Determining the origin of the Mungindi *Fov* strain.

1) Aggressiveness of the Mungindi strain

Virulence of the Mungindi strain (isolate 405/94), one *Fov* reference, and 4 VCG 11 isolates was compared on cotton cultivar Siokra 1-4. Disease severity caused by isolate 405/94 was significantly lower than that caused by either *Fov* reference (24500) or other *Fov* isolates (Figure 10), indicating that the Mungindi *Fov* strain is still at an early stage of virulence evolution. Thus, it may not be a big threat to the industry at the moment; however, it is important to closely monitor its evolutionary process in the field given that a weakly pathogenic isolate of *Fusarium oxysporum* could become more virulent if it can be continuously associated with susceptible cotton plants.

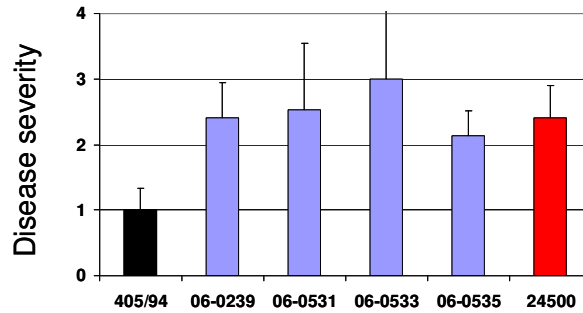


Figure 10. Disease severity caused by the Mungindi *Fov* strain (405/94), *Fov* reference (24500), and four other *Fov* isolates

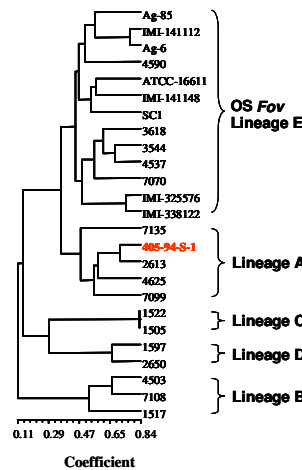


Figure 11. UPGMA dendrogram showing the genetic relationships among the Mungindi *Fov* strain 405/94, Australian native *Fusarium oxysporum* lineages A to E, and overseas *Fov* races 1 to 8

Genetic relationships between the Mungindi *Fov* strain and others

Genetic relationships between the recently identified Mungindi *Fov* strain (new VCG, represented by isolate 405/94, provided by Linda Smith) and 5 lineages of the Australian native *Fusarium oxysporum* and 8 races of overseas *Fov* were assessed using fluorescent AFLPs. Isolate 405/94 is included in the clade of lineage A, suggesting that, similar to the 2 known VCGs, this newly emerging *Fov* strain has also evolved from lineage A (Figure 11). In addition, it is noted that the Mungindi *Fov* strain probably occurs spontaneously in the field since it was represented only by a single isolate and it was not discovered again in a later extensive disease survey in the area.

Outcomes

- Describe how the project’s outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

Detailed briefings of the project’s significant findings, *i.e.* the emergence of novel *Fov* strains in the fields, spatial and temporal variation of *Fov* population associated with cotton cultivars, and variation of virulence among *Fov* genotypes, have been provided to breeders. For sample, several presentations were given in the FUSCOMs. In addition, the

dominant *Fov* genotypes identified in this project were provided to Dr Augusto Becerra, which are now used for the screening of QTLs of Fusarium resistance.

Research outcomes were published in scientific journals. Communications in the forms of oral and poster presentations were made at various seminars, workshops, and conferences.

6. Please describe any:-

a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);

No.

b) other information developed from research (eg discoveries in methodology, equipment design, etc.); and

No.

c) required changes to the Intellectual Property register.

No.

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?

Local origins of *Fov* from lineage A of native *Fusarium oxysporum* and the wide occurrence of lineage A in cotton field soils should be taken into account when disease control strategies are developed since weakly pathogenic lineage A isolates could become more virulent as a result of continuous exposure to susceptible cotton plants. The Australian *Fov* population is genetically diverse, which consisted of 28 genotypes in 2004, and novel pathotypes or genotypes of *Fov* have been found in several regions, e.g. Mungindi. Significant temporal variation of *Fov* population was observed in a Boggabilla field, which has been experimentally proved to be associated with the competitions between *Fov* genotypes on different cotton cultivars. The prevalence of *Fov* in soil declines from the Darling Downs to Moree, suggesting saprophytic ability and aggressiveness of *Fov* are greatly influenced by various soil factors.

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.

No.

(b) for the future presentation and dissemination of the project outcomes.

A presentation will be prepared for the International Congress of Plant Pathology in 2008 in Italy provided the application for travel funding from CRDC is successful.

Two more journals papers will be prepared to disseminate the project outcomes, which are about temporal variation of *Fov*, competitions between *Fov* genotypes, and occurrence and diversity of soil *Fusarium oxysporum* in cotton growing regions.

(c) for future research.

Based on the observation that the prevalence of *Fov* in soil significantly declines from the Darling Downs to Moree, a project was designed to determine the impacts of soil abiotic and biotic factors on saprophytic, aggressiveness, and evolution of *Fov*. This project has been funded by CRDC for the period of 2007 to 2010.

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)

Wang, B., Brubaker, C.L., Tate, W., Woods, M.J., Matheson, B.A. and Burdon, J.J. (2006). Genetic variation and population structure of *Fusarium oxysporum* f.sp. *vasinfectum* in Australia. *Plant Pathology* 55: 746-755 (A PDF copy is provided with this report).

Wang, B., Brubaker, C.L., Thrall, P.H. and Burdon, J.J. (2007). Origin and Evolution of *Fusarium oxysporum* f. sp. *vasinfectum*: A Case Study in Australia. *Journal of Cotton Science* (in press)

Wang, B., Brubaker, C.L., Tate, W., Woods, M.J. and Burdon, J.J. (200X). Evolution of virulence in *Fusarium oxysporum* f. sp. *vasinfectum* using serial passage assays through susceptible cotton. *Phytopathology* (to be accepted as suggested by reviewers)

Wang, B., Brubaker, C.L., Summerell, B. A., Tate, W., Woods, M.J., Matheson, B.A. and Burdon, J.J. (200X). Local origin of two vegetative compatibility groups of *Fusarium oxysporum* f. sp. *vasinfectum* in Australia. *Phytopathology* (submitted)

Wang, B., Brubaker, C.L., Tate, W., Woods, M.J., and Burdon, J.J. (2006). Origin and diversity of *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*) in Australia. Proceedings of the 8th International Mycological Congress (Cairns, 21-25 August, 2006).

Wang, B., Brubaker, C.L., Tate, W., Woods, M.J., and Burdon, J.J. (2006). Genetic diversity of cotton *Fusarium* wilt pathogens in Australia. Proceedings of the 13th Australian Cotton Conference (Gold Coast, 10 August, 2006).

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

No

Part 4 – Final Report Executive Summary

Fusarium wilt of cotton is caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *vasinfectum* (*Fov*). In Australia, 2 *Fov* strains are currently responsible for the disease, each belonging to a distinct vegetative compatibility group (VCG). Previous work showed that the 2 Australian *Fov* strains are genetically related to lineage A of native *Fusarium oxysporum*, suggesting their local origins.

This project aims to: 1) assess the risk of novel *Fov* pathotypes emerging from local *Fusarium oxysporum* population in cotton fields; 2) investigate the evolutionary potential for weakly pathogenic lineage A isolates of native *Fusarium oxysporum* to become more virulent; and 3) determine the competitive ability of different strains or genotypes of *Fov*.

Our work showed that *Fusarium oxysporum* present in the soil of cotton fields consists of 3 major lineages, A, B, and E. The presence of lineage A highlights the likelihood that new strains of *Fov* may emerge in the future. In addition, the difference in lineage composition between uncultivated soils and cotton fields is noticed, with the dominant form of *Fusarium oxysporum* being lineage E in cotton fields but lineage B in uncultivated soils. The evolution of virulence in *Fov* was studied by carrying out the inoculation – re-isolation – re-inoculation infection cycles successively on a susceptible cotton cultivar. A mildly pathogenic isolate of lineage A became more virulent, causing more severe disease symptoms after 10 infection cycles, and importantly, correlated genetic changes were detected in its offspring isolates. This suggests that weakly pathogenic *Fusarium oxysporum* isolates of lineage A can become virulent *Fov* and continuous exposure to susceptible host plants plays an important role in this process.

Based on 350 isolates collected from 6 cotton growing regions in 2002 and 2004, 28 genotypes of *Fov* were identified, with 21 in VCG 11 and 7 in VCG 12. VCG 11 can be further divided into 2 subgroups and subgroup I-B may represent a new strain of *Fov* as all members of this subgroup are incompatible with either known Australian VCG. The occurrence of new *Fov* strains in cotton fields was also detected in other regions like Mungindi. VCG 11 is wide-spread, while VCG 12 is restricted to the Boggabilla area. No genetic structure was found among *Fov* populations at broad geographic scales, but interestingly some structure was found in the Boggabilla area, with the population in two fields being different from the others. In addition, variation in virulence was observed among different genotypes of *Fov*.

Field surveys showed that *Fov* populations may change over time because in one Boggabilla field the dominant *Fov* genotype was 11-A in 2002 but 11-B in 2004 and, furthermore, genotype 11-A was undetectable in 2006. This change is probably driven by the increasing level of resistance in newly released cotton cultivars planted in the field as our glasshouse trials showed that genotype 11-B is more aggressive than genotype 11-A on the tolerant cultivar, suggesting competitions between strains or genotypes of *Fov*.

Fusarium oxysporum occurs at comparable levels in the soil of cotton fields (*Fov* infested or free) in the Darling Downs, Boggabilla, and Moree, however, a clear declining tendency was observed in the frequency of *Fov* in *Fusarium oxysporum* population from the Darling Downs soil to Moree soil. Future studies will focus on soil impacts on *Fov* since, as a soil-borne fungus, both aggressiveness and saprophytic ability are inevitably influenced by soil biotic and abiotic factors. Insights into how life history traits of *Fov* and soil factors interact to determine selection for virulence and persistence of the pathogen would aid developing effective disease management strategies.