2003 BELTWIDE COTTON CONFERENCES, NASHVILLE, TN – JANUARY 6-10

DETECTION, PERSISTENCE AND CONTROL OF *FUSARIUM OXYSPORUM* F. SP. *VASINFECTUM* IN COTTON SEED IN AUSTRALIA

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Abstract

Several methods were employed to detect and identify the pathogen in seed. Seed was plated on agar media in Petrie plates and fungi that grew from the seed were identified by microscopic examination, using taxonomic characters such as spore types and spore formation. Fungi identified as *Fusarium oxysporum (Fo)* in the cotton gin samples were subjected to further testing with DNA Amplification Fingerprinting (DAF) diagnostic tests developed specifically for Australian isolates of *Fusarium oxysporum* f.sp. *vasinfectum (Fov).*

The level of *Fo* was generally between 8 and 11% in seed from infected plants and from ginned cotton, harvested from known infested fields. Some of the seed samples yielded high levels of other *Fusarium* species, mainly *F. equiseti*. *Fo* was not detected in any of the seed, hand picked from healthy plants, even those growing in known *Fov* infested fields.

Fo did not persist in seed stored at room temperature for more than six months although other species of *Fusarium* persisted, but at lower levels than originally detected. The reason for this relatively short persistence of *Fo* is unknown and is somewhat surprising, as the fungus is known to survive in soil for many years. These results indicate that relatively short-term storage may prevent the dispersal of *Fov* in seed lines.

The apparent control of *Fo* in seed by metalaxyl, reported previously, was corroborated by results obtained when two different formulations of the product were compared as seed dressings. Both prevented isolation of *Fo* from seed. The efficacy of metalaxyl is surprising but, as it is routinely used as a component of the planting seed dressing used on cotton in Australia, it is likely to be a factor in reducing the risk of dispersing the pathogen in seed.

Commercial scale fumigation of fuzzy cotton seed stacks with methyl bromide eliminated *Fo* from the seed.

Introduction

Fusarium wilt of cotton, caused by the fungus *Fusarium oxysporum* Schlecht. f.sp. *vasinfectum* (Atk.) Snyd. & Hans. *(Fov)*, was identified in Australia for the fist time in 1993 (Kochman, 1995) almost 100 years after Atkinson (1892) identified the disease in the United States in Alabama. In Australia *Fov* was first isolated from wilted cotton plants collected from the Brookstead/Cecil Plains area on the Darling Downs of Queensland. Fusarium wilt has since been recorded in many commercial cotton production areas of Australia with the exception of the production areas in Emerald in Queensland, Tandou and Hillston in New South Wales and Western Australia.

Initial studies were undertaken to characterise the Australian isolates of *Fov* in order to provide some answers about origin of the pathogen causing the disease. These studies included: pathogenicity tests with differential and local cotton lines; vegetative compatibility groups (VCG) analyses, aesculin hydrolysis tests and volatile compound production tests (Davis *et al.*1996). Hillocks (1992) had reported six physiological races of *Fov*. This was based on reaction of differential hosts that included four cotton and three non-cotton species. Races 1 and 2 originated in the USA, race 3 in Egypt, race 4 in India, race 5 in Sudan and race 6 in Brazil. There is some confusion as to the number of races currently recognised. Apart from the six reported by Hillocks (1992), there appear to be an additional two races (7 and 8) in China (Chen *et al.* 1985) where a further two non-cotton hosts (alfalfa and okra) were added to the differential set. Furthermore, Assigbetse *et al.* (1994) and Fernandez *et al.* (1994) described only three races from a worldwide collection of isolates of *Fov* by using pathogenicity testing in conjunction with RAPD analysis. They constructed a race A that includes isolates previously designated as races 1, 2 and 6. Their races 3 and 4 corresponded with those previously described.

Australian isolates produced reactions equivalent to race 6 on the total set of differential lines. In terms of pathogenicity on the cotton lines in the differential set, they produced reactions equivalent to races 1,2 and 6 or Race A (Davis *et al.*, 1996). VCG analyses and DNA fingerprint tests indicated that Australian isolates belonged to two new VGGs 01111 and 01112, and had different DNA fingerprints to *Fov* isolates from other parts of the world. Hence, it would appear that the Australian isolates developed locally, possibly as a result of wide-scale planting of very susceptible cotton varieties (Kochman *et al.* 1996, 1998).

The pathogen can be seed-borne and may survive within the seed between the kernel and the seed coat (Wickens, 1964). Thus planting infected seed is one of the possible means of introducing *Fov* into new cotton growing areas. Hence we have undertaken a range of studies on detection, persistence and control of *Fov* in cotton seed in Australia. Some of this work has been reported at the Beltwide Cotton Conference in 2001 (Allen & Kochman, 2001). This paper reports some of the results obtained since then.

Materials and Methods

Seed Collection

Seed cotton was hand picked from *Fov* infected and non-infected plants from cotton growing areas in southern Queensland in 2000, 2001 and in 2002. Fuzzy seed was also obtained in 2001and 2002 from a cotton gin that had processed cotton picked from fields where the disease had caused significant losses. The cotton was picked from the more susceptible varieties. Hand picked cotton was ginned with a laboratory gin and a proportion was acid-delinted. Both acid-delinted and fuzzy seed were then used in a number of experiments to determine whether *Fov* could be detected in the sample and what effect the treatments had on survival of the pathogen in the seed.

Detection and Identification of the Pathogen in Seed

Several methods were employed to detect and identify the pathogen in seed. Samples of either 100 or 200 seeds were placed onto 1.5% water agar or onto Nash Snyder selective medium agar for *Fusarium* (Nash and Snyder, 1962) in Petri plates. There were five seeds per plate and 5000 seeds were plated. Agar plates were incubated at room temperature (22-25˚C) for at least seven days on water agar and 10-12 days on Nash Snyder medium. Fungi that grew from the seed were identified by microscopic examination, using taxonomic characters such as spore types and spore formation. *Fusarium* species were identified using international standard identification manuals (Burgess *et al* 1994; Nelson *et al.*1983).

Fungi identified as *Fusarium oxysporum (Fo)* in the cotton gin samples were subjected to further testing. Pathogenic isolates of *Fo* are indistinguishable from non-pathogenic isolates under microscopic examination. DNA Amplification Fingerprinting (DAF) diagnostic tests, specific for Australian isolates of *Fov*, have been developed (Bentley *et al*, 2000) and were used to identify any *Fov* cultures within the *Fo* cultures isolated. The intergenic spacer (IGS) region of the ribosomal DNA (rDNA) was targeted as the basis for a diagnostic test because (i) it is a stable marker, (ii) it is present in multiple copies (allowing more sensitive detection), and (iii) the presence of both conserved and variable regions allows discrimination at different (taxonomic) levels of specificity.

Persistence of *Fo* in Seed

During 2001 ginned, as well as acid delinted seed samples from infected plants were plated onto water agar at regular intervals after hand picking. They were examined microscopically 7-10 days after plating for the presence of *Fo* cultures. Plating ceased two months after no *Fo* was detected. Seed samples were stored in the laboratory in paper bags at room temperature (19-25 ºC).

Control of *Fo* in Seed

Allen & Kochman (2001) reported the complete suppression of the pathogen when seed was treated with metalaxyl. This was surprising as metalaxyl has no reported activity against *Fusarium* species and did not stop growth of the pathogen when incorporated into agar media. Further studies were conducted with two formulations of metalaxyl (Apron XL and Mantle).

Fuzzy seed that had been professionally fumigated with methyl bromide as a requirement for export, was also tested for *Fo* and *Fov* and compared with samples of non-fumigated seed from four samples in 2001 and 12 samples in 2002.

Effect of Varieties of *Fo* in Seed

In 1983 Hillocks reported that infection levels in seed ranged from 2% in a resistant variety to 21% in a susceptible variety. Seed cotton was hand picked from diseased plants of 12 Deltapine varieties, varying in susceptibility to *Fov*, from a trial during April 2001 to determine if similar results would be obtained with Australian isolates of the fungus. Seed was ginned, acid delinted, plated and then inspected for *Fo*.

Results

Detection and Identification of the Pathogen in Seed

Fo was not detected in any of the seed, hand picked from healthy plants, even those growing in known *Fov* infested fields, in any season. Furthermore, in 2002 *Fo* was not detected in seed hand picked from plants showing wilt symptoms in several localities in Queensland and northern New South Wales.

The level of *Fusarium oxysporum* was generally low (8-11%) in seed from infected plants and from ginned cotton, harvested from known infested fields, even though some seed samples yielded high levels of *Fusarium* species.

DNA Amplification Fingerprinting (DAF) for *Fov* Identification

DNA sequence information, unique to the two Australian genotypes of *Fov*, has been identified and used to design PCR primers that specifically amplify DNA only from Australian VCGs 01111 and 01112 of *Fov*. Database searches of DNA sequence information published in Genbank have indicated that there are no matches for these primers with any other organism. The DAF diagnostic test indicated that about 80% of *Fo* colonies isolated from cotton seed were *Fov* and most of these were VCG 01111, the predominant strain in Australia.

Persistence of *Fo* in Seed

Ginned fuzzy cotton, hand picked in May 2000, had *Fusarium* species in 56% of the fuzzy seed and 11% in the acid delinted seed. This seed was used in a number of tests and there was a continuous decline in the level of *Fusarium* in the seed. In October 2000 no *Fusarium* was isolated from the acid delinted seed while the 26% of the fuzzy seed yielded mainly *Fusarium equiseti* (Corda) Sacc. There was no *Fo* recorded in the fuzzy seed either.

Forty seven percent of acid delinted and 93% of fuzzy seed, hand picked in March 2001, yielded *Fusarium* species (Table1). Eleven percent of these isolates were identified as *Fo*. By September *Fo* could not be detected in either delinted or fuzzy seed. Other species of *Fusarium* persisted but at lower levels than originally detected.

Control of *Fo* in Seed

In addition to the seed treatments already reported as being effective in suppressing *Fo* in seed (Allen and Kochman 2001), *Fo* was not detected in any acid delinted seed treated with either formulation of metalaxyl, at the standard rate. Untreated seed yielded 2% *Fo*.

A number of specific aspects of the methyl bromide fumigation studies are commercial-in-confidence so only general results can be provided. Non-fumigated fuzzy seed yielded an average of 10% and 6% *Fo* colonies in 2001and 2002 respectively. Fumigation with methyl bromide was found to eliminate all *Fo* when used at the appropriate concentration for the appropriate length of time. Other species of *Fusarium* did survive fumigation but their number was much reduced in the fumigated seed.

Effect of Varieties of *Fo* in Seed

The proportion of seed that yielded *Fo* varied from 0 in the most resistant varieties to 10% in the most susceptible variety.

Discussion

Many of the results obtained with the Australian isolates of *Fov* in seed cotton are very similar to those already reported overseas. The level of infection in seed from more susceptible varieties ranged from 8 to 11%, within the range of 0.2 to 21% reported in the literature reviewed by Hillocks (1992). Infection levels varied from 0% to 10% in seed from 12 varieties differing in susceptibility to disease, again agreeing with reports in the literature. However, to date the fungus has not been isolated from seed picked from cotton plants not showing wilt symptoms and these data conflict with those reported from Tanzania where infected seed was obtained from plants of a resistant variety not showing wilt symptoms at harvest (Hillocks, 1983). Australian cotton planting seed producers continue to monitor their seed production areas carefully to ensure freedom from *Fov*.

The detection of *Fo* by plating seed is relatively simple but far more work is required to confirm that isolates are *Fov*. This includes pathogenicity tests, which are not always conclusive, VCG analyses and molecular tests. Several DNA diagnostic tests are available for different formae speciales of *Fo*. PCR assays have been developed for the specific detection of *F.oxysporum* f.sp. *ciceris* (Kelly *et al*., 1998) and *F.oxysporum* f.sp. *gladioli* (de Haan *et al*., 2000) based on sequence characterised amplified regions (SCARs); detection of *F.oxysporum* f.sp. *albedinis* (Fernandez *et al*., 1998) and *F.oxysporum* f.sp. *dianthi* (Chiocchetti *et al*., 1999) was based on transposable elements; and a genomic DNA probe has been identified for *F.oxysporum* f.sp. *canariensis* (Plyler *et al*., 1999).

A PCR-based assay for detection of overseas isolates of *Fov* based on small nucleotide differences in the ITS region of the rDNA has been described by Moricca *et al*. (1998). We are doubtful of the suitability of this assay for the specific detection of Australian genotypes of *Fov* because there is little variation within the ITS region of different formae speciales of *F. oxysporum* (Guadet *et al*., 1989; Peterson, 1991; O'Donnell, 1992; Donaldson *et al*. 1995; Bateman *et al*., 1996; Edel *et al*., 1996; Edel *et al*., 2000; Waalwijk *et al*. 1996), and therefore we consider it unlikely that the test will specific for *Fov*. Moricca *et al*. (1998) evaluated the specificity of their PCR assay on a limited number of isolates, including only one isolate from one other form species of *Fo*, three isolates from other species of *Fusarium* and three isolates from other genera. Furthermore, we have found that the primer sequences of Moricca *et al*. (1998) match DNA sequences in many other formae speciales of *Fo* (based on BLAST searches of sequence information published in Genbank). The lack of intraspecific variability within the ITS region of the rDNA, combined with the high degree of genetic diversity within *Fo*, requires a much more thorough evaluation of primer specificity to ensure the specificity of the assay.

The problems with the tests based on the ITS region led Bentley *et al* (2000) to investigate the intergenic spacer (IGS) region of the rDNA as the basis for our diagnostic test. The IGS region of rDNA was targeted because (i) it is a stable marker, (ii) it is present in multiple copies (allowing more sensitive detection), and (iii) the presence of both conserved and variable regions allows discrimination at different (taxonomic) levels of specificity.

Information obtained on genetic diversity among Australian isolates of *Fov* has been used in the development of polymerase chain reaction (PCR)-based DNA diagnostic tests for the rapid detection of *Fov*. These tests are able to differentiate accurately Australian isolates of *Fo* and the two *Fov* VCGs from international isolates of *Fov* when their DNA is extracted from colonies grown in pure culture. Further development and validation is currently being conducted to ensure that the diagnostic tests can be used on DNA extracted directly from seed, plant material or soil.

There appears to be little published information on persistence of *Fov* in seed. The data obtained from our experiments indicate that *Fo* could not be isolated from seed stored at room temperature for six months although other *Fusarium* species continued to be isolated. The reason for this relatively short persistence of *Fo* is unknown and is somewhat surprising, as the fungus is known to survive in soil for many years. A possible explanation is that the saprophytic phase of the *Fos'* life-cycle, which is likely to be very important for their survival in soil, may not occur in seed stored in a dry environment. The fact that *Fo* was eliminated by methyl bromide fumigation when other *Fusarium* species were reduced in number, lends further support to the theory that *Fo* may have an inferior survival potential in seed than other species of this genus. These results indicate that relatively short-term storage may prevent the dispersal of *Fov* in seed lines.

The apparent control of *Fo* in seed by metalaxyl, reported by Allen and Kochman (2001), was corroborated by results obtained when two different formulations of the product were compared as seed dressings. Both prevented isolation of *Fo* from seed. The efficacy of metalaxyl is surprising, but as it is routinely used as a component of the planting seed dressing used on cotton in Australia, it is likely to be a factor in reducing the risk of dispersing the pathogen in seed.

Acknowledgements

The authors would like to acknowledge the financial support of research projects by the Farming Systems Institute of the Agency for Food and Fibre Sciences in the Queensland Department of Primary Industries, the Cotton Research and Development Corporation, the Australian Cotton CRC, the CRC for Tropical Plant Protection. The assistance of many cotton growers, consultants and other industry representatives has been very valuable in setting and reviewing research directions. The technical assistance of Mr M Mette, with some of the seed testing, is also acknowledged.

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Table 1. The percentage of seed, yielding *Fusarium* species, when plated at various intervals after harvest in 2000 and 2001.

	2000					2001			
	Acid delinted		Fuzzy			Acid delinted		Fuzzy	
Month	*Fusarium* species	*Fo*	*Fusarium* species	*Fo*	Month	*Fusarium* species	*Fo*	*Fusarium* species	*Fo*
May	11	9	56	11	March	47	11	93	13
October	1	0	28	0	May	25	5	82	7
Jan 2001	0	0	25	0	August	26	2	76	1
					September	26	0	80	0
					November	24	0	80	0