

Verticillium and Fusarium Wilts of Cotton: Molecular Genetic Markers for Disease Resistance

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1. Introduction

1.1 Verticillium and Fusarium Wilt In Cotton

Verticillium wilt and Fusarium wilt, vascular diseases caused by the soil-borne fungal pathogens *Verticillium dahliae* and *Fusarium oxysporum* f.sp. *vasinfectum*, respectively, are among the most important diseases of cotton (*Gossypium* spp.), with the potential to cost the cotton industry millions of dollars in lost production. These fungi colonise the plant roots and penetrate the vascular tissues, where they proliferate in the vascular system and are eventually distributed throughout the plant. Plants infected with either *Verticillium* or *Fusarium* display similar symptoms of chlorosis and necrosis of leaves, defoliation, stunting or plant death. Cross-sections of the stem reveal a brown or black centre which is due to the formation of melanised products in the infected vascular system. However, chlorosis in Fusarium wilt tends to be in patches and vascular browning is more pronounced than in Verticillium wilt. In general, the symptoms of Fusarium wilt are much more severe and result in greater crop losses (Bell, 1992a and 1992b).

1.2 Molecular Marker Technology

The most effective and durable defence strategy against Verticillium and Fusarium wilt is the breeding of resistant cotton cultivars. At present, the Australian cotton industry has managed to largely control damage caused by Verticillium wilt through the use of Verticillium wilt-tolerant cultivars. However, there is always the possibility that new strains of *Verticillium* may arise which can overcome the resistance currently offered by these cultivars. On the other hand, Fusarium wilt was first identified in Australian cotton fields in 1993 and it constitutes a major concern to the Australian cotton industry as new cultivars specifically resistant to this disease have yet to be developed. It is therefore essential that new sources of host plant disease resistance are identified within the *Gossypium* germplasm and incorporated into the existing cotton breeding programs. Durable disease resistance is usually achieved through 'gene pyramiding' ie. breeding a number of resistance genes into a single cultivar. However, traditional plant breeding techniques present difficulties when it comes to testing plants for the presence of multiple resistant genes. Molecular marker technology, which in this case would involve the identification of unique DNA fragments (molecular genetic markers) associated with genes for resistance to Verticillium wilt and/or Fusarium wilt, will enable the development of rapid screening assays for disease resistant germplasm. In these assays, the mere possession of the molecular marker by young seedlings will signify the presence of enhanced resistance, and thereby eliminate the need for the selection of disease resistant plants by pathogenicity testing.

1.3 Research Aims

The primary aim of the research projects described here is to identify molecular genetic markers linked with resistance to *Verticillium* wilt and/or *Fusarium* wilt in cotton. In order to find molecular genetic markers linked with disease resistance, a plant population is required in which the genes for disease resistance or susceptibility segregate amongst the individuals (F2 progeny of a cross between resistant and susceptible parents). This population is subjected to pathogenicity tests and individuals are scored for their response to the disease. Molecular genetic markers identified within the same population whose segregation correlates with the disease data represent molecular markers linked with either disease resistance or susceptibility. Progeny from the intraspecific crosses between the *Gossypium hirsutum* cultivars Siokra 1-4 (*Verticillium* wilt-susceptible) x Sicala V-1 (*Verticillium* wilt-tolerant), and CS 50 (*Verticillium* wilt-susceptible) x Acala Royale (*Verticillium* wilt-tolerant), are being used to identify molecular markers linked with *Verticillium* wilt (Lyon *et al.*, 1996). The identification of *Fusarium* wilt markers will be carried out in progeny from the crosses between Siokra 1-4 (*Fusarium* wilt-susceptible) x CS 189⁺ (*Fusarium* wilt-tolerant) and Siokra 1-4 (*Fusarium* wilt susceptible) x MCU-5 (*Fusarium* wilt-tolerant).

2. Results and Discussion

2.1 Development of a Wilt Disease Grading System

Disease grading methods for *Verticillium* wilt in cotton that have been reported in the literature arbitrarily give certain plant disease symptoms greater weight than others. We chose instead to develop a comprehensive disease grading system, based partly on the systems of Staffeldt and Fryxell (1955) and Wilhelm *et al.* (1974), which scores each disease symptom independently and keeps clear records of the disease response of each plant throughout infection. This disease grading system relies on three characters of infected plants: leaf symptoms, plant height and vascular discolouration of the stem (Table 1). Leaf symptoms and plant height are recorded at regular intervals until the mature plants are cut following the collection of seeds, at which time vascular discolouration of a section at the base of the plant stem is also recorded. An average of the leaf grading recorded at different intervals is taken as the overall leaf grading for each plant.

Disease assays for *Verticillium* wilt were conducted on approximately 250 F2 plants from each of the two *G. hirsutum* crosses (Siokra 1-4 x Sicala V-1 and CS 50 x Acala Royale). Disease assays conducted on the parental cultivars had shown that leaf, vascular and height symptoms were differentially expressed between the resistant and susceptible parents and that plant height was the clearest indicator of disease resistance. Disease symptoms in the F2 segregating progeny of both crosses were variably expressed. The distribution of plants in the F2 population with regards to vascular discolouration and plant height are shown in Figure 1a and 1b, respectively. As with the parental analysis, plant height was found to be the most reliable disease symptom since this data was not based on a subjective assessment, but rather on accurate measurement. Analysis of the disease data for both crosses revealed a continuous variation in all the disease symptoms, which suggests that the *Verticillium* wilt disease resistance character is probably due to the action of several genes.

Table 1: Disease Grading System

Leaf Symptoms	Vascular Symptoms	Height Symptoms
0 - no external leaf symptoms	0 - no vascular symptoms	Accurate plant height measurements recorded at regular intervals
1 - slight leaf wilting or yellowing	1 - brown discolouration in core of stem	
2 - moderate leaf yellowing and necrosis, slight defoliation	2 - black discolouration in centre core tissue only	
3 - severe leaf yellowing and necrosis, appreciable defoliation	3 - black centre and discolouration extended into surrounding tissue	
4 - dead plant, complete defoliation	4 - black discolouration throughout stem section	
	5 - completely black stem and plant death	
<u>0 or 1</u> average leaf symptom classification - resistant <u>3 or 4</u> average leaf symptom classification - susceptible	<u>0 or 1</u> - resistant <u>4 or 5</u> - susceptible	Extremes of plant height were regarded as resistant or susceptible

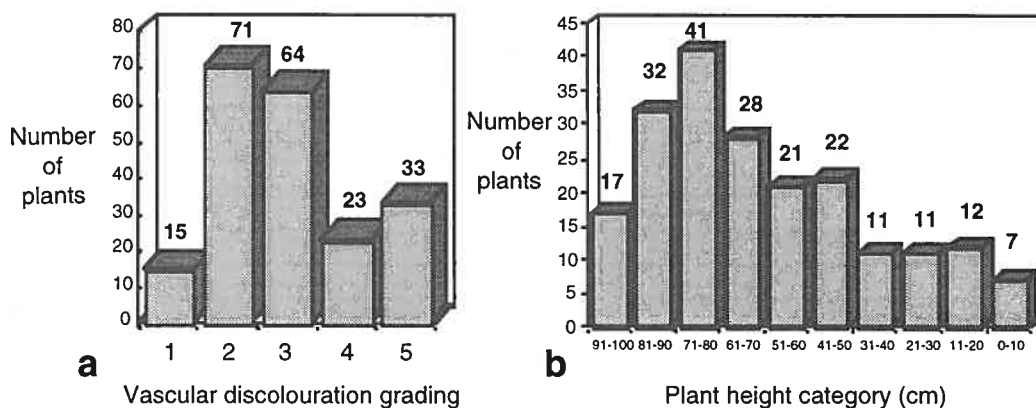


Figure 1: a) Frequency distribution of *Verticillium*-infected F2 plants from the cross Siokra 1-4 x Sicala V-1, graded according to vascular discolouration. b) Frequency distribution of final height of *Verticillium*-infected F2 plants from the cross Siokra 1-4 x Sicala V-1.

2.2 Development of a Molecular Assay for Wilt Disease

Fusarium infection of cotton plants during disease grading assays might cause the death of the host plant and therefore result in an undesirable loss of plant material required for generational studies. A plant cloning method has therefore been developed which enables the production of viable clones of each individual plant that can be retained in an uninfected state for subsequent seed production (Figure 2, a and b). An alternative disease-grading method is also being tested to assess *Fusarium* wilt. This method utilises a fungal-specific DNA amplification process, with primers for the polymerase chain reaction (PCR) designed to amplify DNA from the *Fusarium* organism as it infects inoculated cotton plants (Figure 2, a-d). The disease grading system for this method is based on the fact that infecting fungi are able to move further up the vascular system of disease susceptible plants due to the delayed defence response of these plants (Figure 2, g) relative to resistant plants (Figure 2, f). Tissue samples of 2.5 cm of stem are excised from above the inoculation point (Figure 2, e). The 2.5 cm stem is dissected into 5 mm pieces and each piece is used to extract DNA (Figure 2, h). The DNA template is used to set up a PCR amplification with fungal-specific primers (Figure 2, i), and amplification products are analysed by agarose gel electrophoresis (Figure 2, j). Presence of a DNA band at about 400 base pairs indicates the presence of the *Fusarium* pathogen in the plant tissue and hence disease susceptibility, whereas the absence of the band indicates the absence of the fungus in the tissue and hence relative disease resistance.

2.3 Comparison of Molecular Marker Techniques

To investigate the potential for detecting polymorphic markers in cotton, four molecular marker techniques: random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), random amplified hybridisation microsatellite (RAHM) (Cifarelli *et al.*, 1995), inter-simple sequence repeat (ISSR) (Zietkiewicz *et al.*, 1994) and amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), were tested on cultivars CS 50 and Acala Royale. All four techniques are based on PCR amplification and target multiple loci in the genome. RAPD analyses are performed on genomic DNA templates primed with a random oligonucleotide primer, resulting in the amplification of numerous discrete DNA fragments. RAHM combines RAPD amplification and screening for simple sequence repeats (microsatellites). ISSR amplification utilises primers designed to target a subset of microsatellites and results in the amplification of regions between closely-spaced microsatellites. The AFLP approach relies on the selective PCR-amplification of restriction fragments derived from the genome.

The level of polymorphism (genetic difference) detected between the two cultivars was 6.1% for AFLP, 6.8% for RAPD, 14% for ISSR and 27.6% for RAHM. Although a high percentage of polymorphism was displayed by the microsatellite-based techniques, the high degree of mutation within microsatellites results in instability of these markers from generation to generation and is a potential drawback for plant breeding applications. However, the polymorphisms observed with the RAPD and AFLP techniques are due to base substitutions within the genome which are more genetically stable in subsequent generations. RAPD was used initially since it was the most established of all the techniques tested and potentially offered rapid results for marker-assisted breeding.

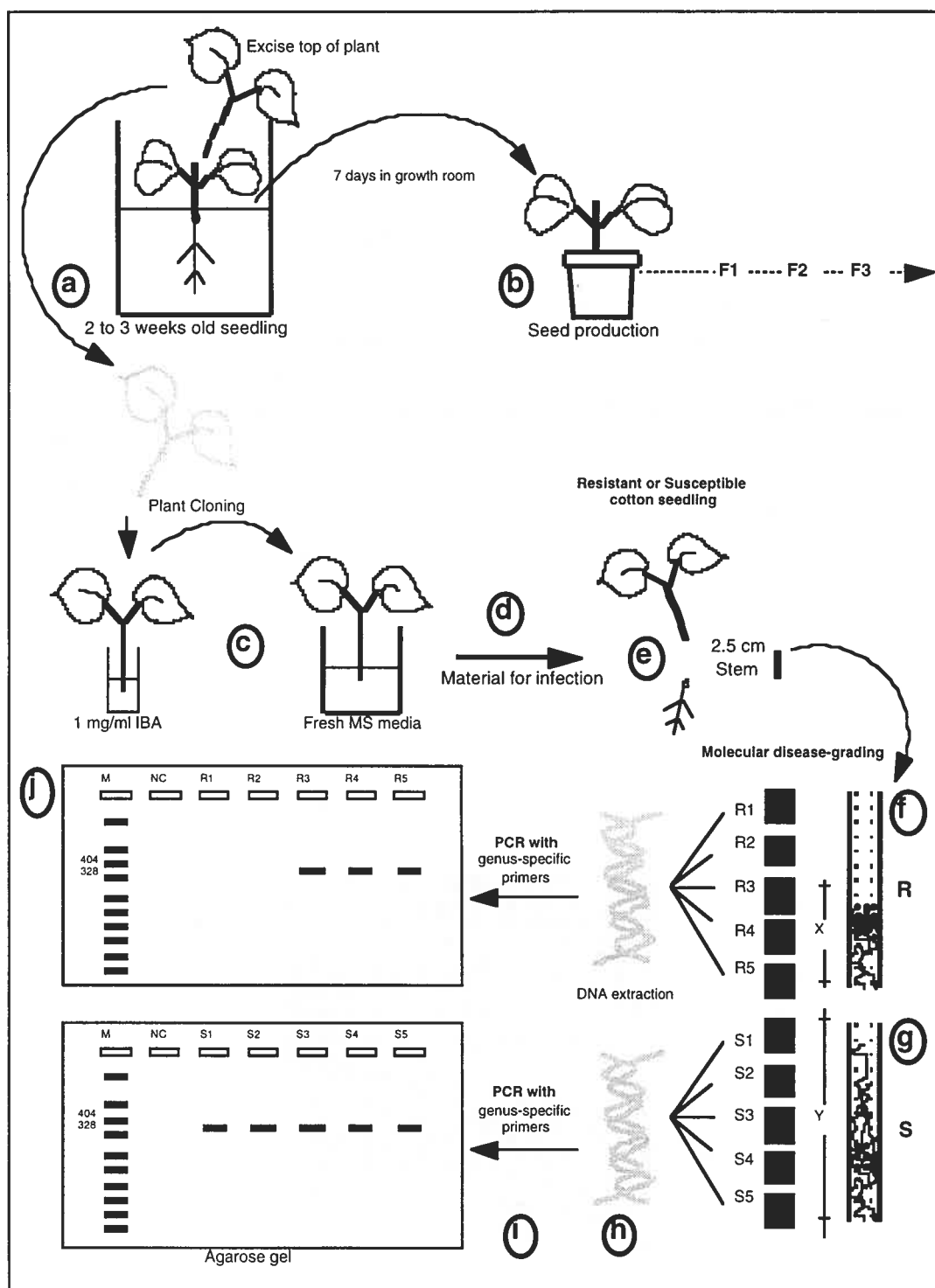


Figure 2: Molecular assay for wilt disease. One of the two cotton plant clones is transferred to a soil pot to preserve a viable (uninfected) plant for subsequent DNA analysis or seed harvest (b). The other plant clone (c) is used for infection with *Fusarium* (d). The PCR DNA-based assay using genus-specific primers is conducted (e-j). The presence of a DNA band at about 400 base pairs indicates the presence of the fungus in the plant tissue and hence disease susceptibility, whereas the absence of the band indicates the absence of the fungus in the tissue and hence disease resistance.

2.4 RAPD Analyses of Wilt Resistant Cotton Cultivars

RAPD was carried out on the two parental cultivars Siokra 1-4 (*Verticillium* wilt-susceptible) and Sicala V-1 (*Verticillium* wilt-tolerant) using 340 random primers. About 3000 amplified loci were generated, of which 96 were polymorphic between the parents. This represents 3% polymorphism between the two cultivars. These 96 polymorphisms were further examined to determine whether they are linked to disease resistance, by conducting RAPD on the DNA of F2 plants pooled on the basis of vascular discolouration and leaf symptoms (See Figure 3 legend). Two markers, generated by primers OPG-17 (Figure 3) and OPV-18, indicated linkage to disease resistance and susceptibility, respectively. DNA from individual F2 plants was tested by RAPD and Southern analysis to examine the segregation of the candidate markers in the F2 population. Statistical analysis of these results indicated that the markers were linked to *Verticillium* wilt disease, although the marker was not present in every plant.

A similar study examined the CS 50 (*Verticillium* wilt-susceptible) and Acala Royale (*Verticillium* wilt-tolerant) cultivars. Approximately 2000 amplified loci were generated, of which 148 were polymorphic between the parents. This represents 7% polymorphism between the two cultivars. These 148 polymorphisms were further examined by RAPD on the DNA of F2 plants, pooled on the basis of vascular discolouration and plant height symptoms, to determine whether they were linked to disease. Although two markers, generated by primers OPL-08 and OPU-16 appeared to be linked with disease resistance, they failed to amplify reliably and would not be suitable for use in marker-assisted breeding. Many of the polymorphisms observed using RAPD analysis displayed poor reproducibility and had to be dismissed. It was therefore decided to adopt the more robust AFLP technique.

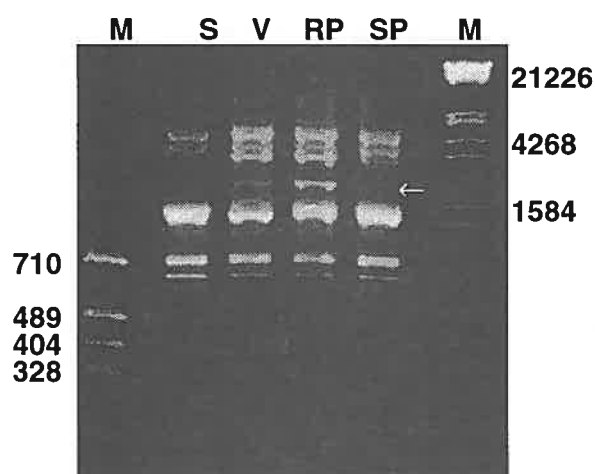


Figure 3: RAPD-PCR analysis using primer OPG-17 on Siokra 1-4 (S), Sicala V-1 (V), resistant (RP) and susceptible (SP) F2 pooled DNA. The arrow indicates the polymorphic band which is present only in the resistant parent and resistant F2 DNA pool. This polymorphism is proposed to be linked with resistance to *Verticillium* wilt. Size markers (M) are shown in base pairs. DNA from F2 plants in the extremes of susceptibility and resistance were used to constitute DNA pools according to the principle of bulked segregant analysis (Michelmore *et al.*, 1991). This principle involves combining (pooling) DNA from individual F2 plants selected to have a similar genomic region for the character of interest (disease resistance or susceptibility) but with random genomic regions at all other sites.

2.5 AFLP Analyses of Wilt Resistant Cotton Cultivars

AFLP Optimisation with the Leaf Shape Trait

The parental cultivars Siokra 1-4 (okra leaf) and Sicala V-1 (normal leaf) also differed according to the leaf shape trait. This trait segregated in the F₂ population to yield normal, intermediate and okra leaf shapes in a 1:2:1 ratio. The leaf shape character was used as a model system to optimise the AFLP marker technology since this character was clearly due to a single gene, unlike resistance to *Verticillium* wilt. The okra and normal leaf shape pools had been examined by RAPD analysis using 340 random primers, but none of the identified polymorphic markers were found to be linked with leaf shape. AFLP analysis was conducted on leaf shape pools using 64 primer combinations (*EcoRI* and *MseI*) from the AFLP™ Plant Mapping kit (Perkin-Elmer, USA). Some 1660 loci were generated with five reproducible polymorphisms. These results were obtained by automated analysis of the AFLP reactions using the Genescan® software (Perkin-Elmer, USA). Cloning of the five identified AFLP polymorphisms is underway. These markers will be confirmed by AFLP testing on individual F₂ plants.

AFLP Analysis for Verticillium Wilt Resistance Markers

AFLP analysis was performed on parents and F₂ progeny of the CS 50 and Acala Royale cross to identify molecular markers linked with disease resistance. A total of 1700 loci were generated using 64 primer combinations of which 104 were polymorphic. The polymorphisms generated were examined in the F₂ pools and two candidate markers have been identified so far. These markers will be confirmed by AFLP testing on individual F₂ plants. AFLP analysis will also be conducted on parents and F₂ progeny of the Siokra 1-4 x Sicala V-1 cross to identify molecular markers linked with disease resistance.

3. Conclusion

The search for molecular markers linked with cotton plant resistance to fungal wilt disease was previously hindered by the lack of a reliable disease scoring procedure. A robust grading system has now been developed for assessing the symptoms of *Verticillium* wilt under controlled conditions. A similar system is being developed for *Fusarium* wilt, with the added consideration that *Fusarium* wilt is more severe than *Verticillium* wilt and may cause the undesirable loss of plant material. In addition, a molecular assay for wilt disease is being developed which will facilitate the rapid grading of wilt-infected plants.

Although RAPD analysis tends to suffer from a lack of reproducibility, one RAPD marker was identified which is linked with resistance to *Verticillium* wilt, while a second RAPD marker was linked with susceptibility to the disease. AFLP analysis, which showed much greater reliability than RAPD, identified five possible markers linked with leaf shape and two candidate markers linked with resistance to *Verticillium* wilt. These results are being confirmed by AFLP analysis of individual F₂ plants to check the segregation of the markers within the population. Details of the results will be presented in a poster at the Conference.

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