Verticillium Wilt of Cotton: Application of Molecular Genetic Techniques in Fingerprinting and Gene Cloning

D. S. Multani[1], M. K. Hill[1,2], J. R. Ramsay[1] and B. R. Lyon[1,2]

[1]School of Biological Sciences A12, The University of Sydney, NSW 2006
[2]Cooperative Research Centre for Sustainable Cotton Production

1. Introduction.

The incidence of Verticillium wilt disease in cotton is increasing and costs the industry millions of dollars annually in lost yield. Information about the pathogenic races of *Verticillium dahliae* prevalent in the cotton growing areas of Australia, their mode of spread and their extent of field persistence is lacking. Furthermore, although a few cotton cultivars developed by the CSIRO have shown some degree of tolerance to Verticillium wilt in the field, little is known about the number and kinds of defence genes that they possess.

With the advent of new molecular genetic techniques, it is now possible to identify the particular pathogen strain in an outbreak of plant disease, to characterise it, and potentially, to isolate genes associated with pathogenicity. Similarly, such techniques can also be employed to differentiate between plant cultivars and progeny in plant breeding programs and in the identification of genes implicated in improved plant tolerance to diseases. The sensitivity of these techniques rests in their ability to detect the rare or subtle differences that exist between the genes of one individual and another. In the present investigation, we have applied Polymerase Chain Reaction (PCR), Random Amplified Polymorphic DNA (RAPD) -PCR and gene cloning techniques to strains of *V. dahliae* isolated from cotton plants, and to cotton cultivars tolerant to Verticillium wilt, with the aim of achieving a better understanding of this pathogenic disease in cotton.

2. *Verticillium dahliae* in Australian cotton.

2.1. Amplification of variable DNA regions from *V. dahliae*.

An important advance in the identification of organisms using molecular genetic methods involves the development of a technique of DNA amplification known as the Polymerase Chain Reaction. The PCR is an *in vitro* procedure for the enzymatic synthesis of DNA, which uses two small DNA pieces known as primers that specifically anneal with opposite strands of a DNA molecule flanking a region of interest. A repetitive series of cycles involving DNA strand separation, primer annealing, and the synthesis of copy DNA results in the production of numerous identical DNA fragments. The amplification is dramatic because the amplified products of one cycle serve as templates for the following reactions and thus after 20 cycles of the PCR, a 10^6 -fold amplification (2^{20}) is achieved.

We have employed the PCR to amplify a variable DNA region, known as the Internal Transcribed Spacer (ITS), from a number of suspected *V. dahliae* strains isolated from cotton plants grown in N.S.W. and Queensland using specific primers. As expected, we found that all of the strains exhibit an amplification product of characteristic size which has been shown by researchers elsewhere to indicate the presence of members of the Genus *Verticillium.*

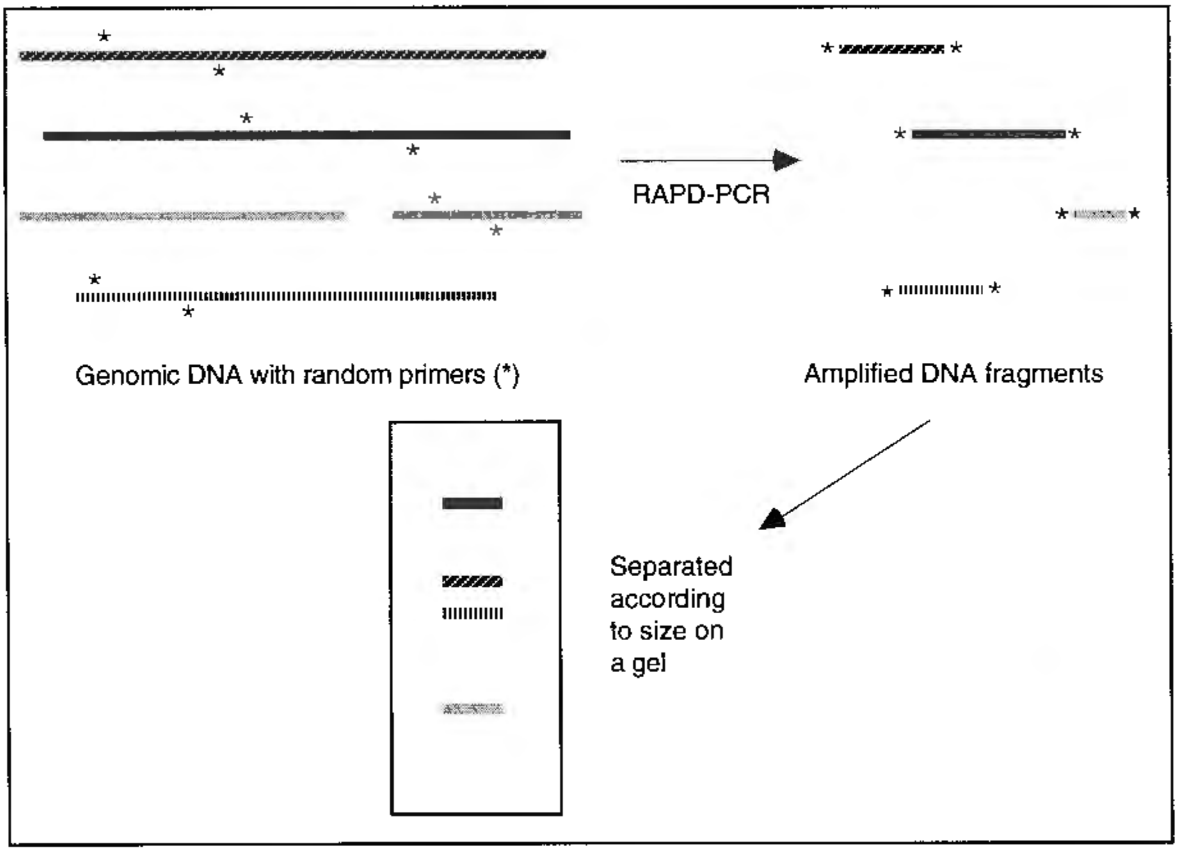

Figure 1. Diagrammatic representation of the Random Amplified Polymorphic DNA (RAPD) -PCR and separation of amplified DNA fragments on a gel.

2.2. Amplification of random DNA regions from *V. dahliae.*

As PCR amplification of the ITS variable regions does not distinguish between individual strains or populations of *V. dahliae*, we chose to use the relatively new RAPD-PCR technique. The RAPD assay is a variant of the PCR in which a single primer of random sequence is used (Figure 1). With any given primer a number of amplification products usually result, and any products that are not present amongst the amplification products of other closely related individuals are said to be polymorphic. The use of a large number of different primers allows the detection of numerous polymorphic fragments which enables a comparison of the genetic relatedness of individuals. Strains with many common fragments are considered to be more genetically alike than those with few common fragments.

Twenty-six random primers from a total of 80 that were tried worked well on *Verticillium* DNA, with each primer producing a unique pattern of bands of various sizes. Eight of the 26 primers produced many amplification products that were common between strains, together with numerous polymorphic products that were unique to one or only a few strains. The markers generated by these eight primers were sufficient to distinguish between five strains of *V. dahliae* from N.S.W. and four strains from Queensland. Analysis of genetic relationships suggests that strains of *V. dahliae* isolated from diverse sites in both N.S.W. and Queensland are quite closely related and are possibly derived from a common progenitor strain. However, each strain possesses a discrete RAPD profile (fingerprint) which would enable it to be identified in surveys designed to investigate the epidemiology of these fungi in cotton crops.

2.3. An epidemiological survey of *V. dahliae* in Australian cotton.

In order to gather more information about the prevalence, survival and mode of spread of the *Verticillium* fungus, two fields of cotton at Narrabri and one at Warren were selected for collection of Verticillium wilt infected plants. Samples were collected from 25 marked locations from each field, and samples were assigned a number that could be traced to a specific location in a given field so that repeat samplings can be made during the next cropping season. In addition to these 75 samples, five samples taken at random from Warren, 13 collections representing Boggobilla, Breza, Boggolani, Cryon, Bourke, Croopa Creek, Baau Baa, Boomi, Barren Junction and Maules Creek areas of N.S.W. and six samples representing Leopard, Plantation, Bilorey and Snow farms of St. George, Queensland, were also included in this study. *Verticillium* fungus was isolated from infected cotton stems and cultures were raised on ethanol agar media supplemented with antibiotics. Variations in growth pattern among the various isolates was observed indicating the existence of a complex population structure of *V. dahliae* in these cotton growing areas. To date, we have used PCR analysis using known primers for amplifying ITS regions and RAPD-PCR analysis on nine representative samples. This work, although at an early stage, shows promise in achieving the stated objectives.

2.4. Artificial infection of cotton with *V. dahliae.*

Most of the studies conducted on Verticillium wilt of cotton in Australia have been surveys of the extent of damage to the cotton crop. Although we have successfully differentiated isolates of *V. dahliae* using RAPD-PCR, pathogenicity tests remain the only means of determining the pathological classification (races) of fungal strains present in diseased cotton plants. We therefore aim to determine the reaction of individual fungal strains on a set of cotton cultivars, and to date, we have standardised an artificial technique of induction of Verticillium wilt and inoculated six identified strains of *V. dahliae* on Sicala V1 and Siokra 1-4 plants. In addition to establishing a system for the classification of pathological races of *V. dahliae*, this work may assist in the identification of plant genes which impart tolerance to particular strains of the fungus.

3. Analysis of Verticillium wilt-tolerant cotton varieties.

3.1. Genetic fingerprinting of cotton cultivars.

Improved cotton cultivars developed through traditional plant breeding procedures are mostly selections from intervarietal crosses and backcross programs. As these plants inherit much common genetic material, knowledge of the genetic similarity between individuals is useful in facilitating efficient utilisation of germplasm sources. For example, the breeder can use this information in selecting diverse parents to cross in hybrid combinations to maximise the expression of hybrid vigour. Futhermore, most of the desirable agronomic traits such as yield, quality and resistance to diseases and insect pests are governed by many genes, which are attributing in an additive manner. These traits are known as quantitative traits and genes controlling them as Quantitative Trait Loci (QTL). Pyramiding of QTLs into a variety is always a major objective of every plant breeder, but it is difficult to achieve as such genes are normally scattered throughout the plant genome rather than being grouped in a defined region. However, pyramiding of QTLs does become possible if the desired genes are tightly linked with some selectable markers. While commonly used morphological traits (phenotypic markers) for selection in the breeding process are limited in number and are highly influenced by environmental factors, molecular genetic markers, such as those identified by RFLP (Restriction Fragment Length Polymorphism) and RAPD-PCR analysis, can provide more extensive information in the form of genetic fingerprints for breeding lines and released cultivars. These genetic markers could be employed in the identification and characterisation of varieties and in testing the purity of F1 hybrids, and may also be helpful in providing a basis for selection of more divergent individuals and in tracking and manipulating QTLs in the ongoing breeding programs.

Twelve cultivars and a breeding line of *Gossypium hirsutum*, together with an American Pima cotton (*G. barbadense*) cultivar were screened for RAPD markers with a total of 80 random primers. A list of these cultivars and their reactions to Verticillium wilt and Bacterial blight is given in Table 1. Thirty DNA primers worked well on these fourteen lines and 23 primers produced polymorphic banding profiles for Pima S7 cultivar from that of *G. hirsutum* cultivars, confirming that Pima cotton S7 is relatively more divergent from Sicala, Siokra, CS and Deltapine *G. hirsutum* cultivars.

Cultivar	Verticillium Wilt*	Bacterial Blight#	Markers
Sicala V1	T	I	2
Sicala V2	T	I	-
001-1223	S	I	5
Siokra 1-4	S	I	2
Siokra L22	S	I	-
Siokra L23	S	I	-
Siokra V15	T	I	2
CS 7S	S	I	-
CS 8S	T	I	1
CS 50	S	I	14
CS 189+	T	I	1
DP90	S	S	20
S295	-	I	7
Pima S7	T	S	69

* T = tolerant, S = susceptible. # I = immune, S = susceptible.

Table 1. List of cotton cultivars studied, their reactions to Verticillium wilt and Bacterial blight, and number of cultivar-specific RAPD markers.

A total of 312 RAPD markers (bands observed on gels) were identified in 13 *G. hirsutum* cultivars using the 30 primers. Over 40 per cent of the banding profile for each primer was found to be common, indicating more than 40% genetic similarity in all 13 *G. hirsutum* cultivars studied. In pairwise comparisons, Sicala V1 and Sicala V2, which are offspring of the same double-cross, were genetically more than 90% similar, and the genetic similarity of Siokra L22 and Siokra L23, and CS 7S and CS 8S, also reflected the same trend. Thus, the relationships among the cultivars concluded from this study were in accordance with the pedigree information. An added advantage of the RAPD results is that it is now possible to differentiate closely-related varieties based on genetic fingerprints. Sicala V1 and Sicala V2 differ with respect to one band (present in one and absent in other) when using the OPM-4 primer (Figure 2). Similarly, Siokra L22 can be distinguished from Siokra L23 using two different primers and CS 7S from CS 8S by using any of five primers. The banding pattern of each individual cultivar for a given primer was then compared to identify cultivar specific markers. Nine out of 13 *G. hirsutum* cultivars can be characterised individually based upon cultivar-specific RAPD markers (Table 1). Deltapine 90 and CS 50 are easily distinguishable from other cultivars with a wide array of cultivar-specific markers (Figure 2).

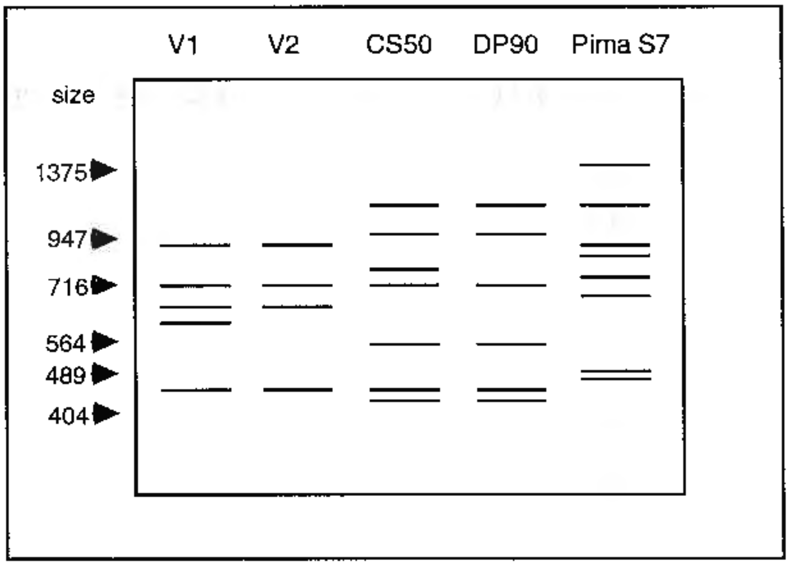

Figure 2. Banding profile of five different cotton cultivars produced with the OPM-4 random primer using the RAPD-PCR technique.

RAPD analysis of released cultivars also depicts the contribution made by a specific parent. The improved cultivars with common parentage tended to group with each other (like Sicala V1 and Sicala V2) or with one of their parents (L22, L23 and CS50). This type of RAPD analysis can therefore inform the breeder of the genetic basis of any outcome and thus provides useful insights for the design of future breeding strategies.

The two Verticillium wilt-tolerant lines Sicala V1 and Sicala V2 are polymorphic from CS 8S, a wilt-tolerant line derived from the same cross, with respect to 8 RAPDs generated by five primers. Meanwhile, another wilt-tolerant cultivar CS 189+ differs from 001-1223, a sister breeding line selected from the F2 generation of the same cross yet susceptible to Verticillium wilt, with respect to 67 RAPDs generated by 21 primers. As these RAPD products segregate in Mendelian fashion, they can be effectively used as genetic markers. The F2 populations or F3 families derived from crosses of tolerant and susceptible parents will be analysed to establish associations between RAPD markers and genes imparting tolerance to Verticillium wilt. This will help in marker-aided selection and in the tailoring and pyramiding of potential resistance genes during the development of improved wilt-tolerant cotton cultivars.

3.2. A targeted search for wilt-tolerance genes in cotton.

Plants command an array of inducible defence responses which can be stimulated by microbial pathogen attack. The induction of the defence response involves substantial changes to the pattern of plant gene expression, and results in the accumulation of numerous gene products including antimicrobial hydrolases such as β-1,3 glucanase and chitinase, cell wall modifying enzymes such as aniomic peroxidase and enzymes belonging to biosynthetic pathways that manufacture defensive chemicals. Molecular genetic techniques have been used to isolate a number of genes associated with host resistance to pathogenic diseases in a variety of plants. The aim of our research is to isolate genes that are expressed in response to *V. dahliae* infection from Verticillium wilt-tolerant cotton varieties, and to develop these genes for use as molecular markers in the screening of plant breeding material for improved wilt-tolerance.

Initial gene analysis work has concentrated on the wilt-tolerant variety Sicala V1, and is directed towards the construction of a cDNA library which represents a collection of all of the genes that are induced as part of the plant's defence response to infection with *V. dahliae*. Characterisation of the genes by DNA sequencing and comparison with genes of known function from other plants may indicate the role of these genes in the defence response of cotton. Genes exhibiting rapid and/or intense levels of expression in Verticillium wilt-tolerant varieties will be selected for further analysis as they may play a part in the superior disease tolerance of these varieties.

4. Conclusion.

Polymerase Chain Reaction (PCR) technology has proved to be extremely useful in confirming the presence of *Verticillium dahliae* in both infected cotton plant tissue and isolated culture, and in identifying strains of *V. dahliae* isolated from cotton growing areas in N.S.W. and Queensland. Genetic relationship analysis of these strains suggests that they are quite closely related and possibly derived from a common progenitor strain. Efforts will be strengthened to investigate the population structure and genetic diversity of *V. dahliae* in cotton by using the RAPD-PCR technique. *Verticillium* cultures collected randomly or intensively from fields in various Australian cotton growing areas have been prepared for a detailed molecular analysis of the pathogen, and the epidemiological data obtained will be used to identify and clone gene markers associated with *V. dahliae* strain pathogenicity/virulence.

Our results have also confirmed the practicality and applicability of using RAPD markers to establish genetic fingerprints of cotton cutivars developed by the CSIRO cotton breeding program. Closely related cultivars such as Sicala V1 and Sicala V2, Siokra L22 and Siokra L23, and CS 7S and CS 8S, are readily discriminated, and nine *G. hirsutum* varieties presently under cultivation in Australia can be characterised individually based upon cultivar-specific RAPD markers. The future use of the RAPD-PCR technique will aim to establish linkages between molecular genetic markers and Verticillium wilt-tolerant genes in selected cotton cultivars, and this will be augmented by the isolation of genes from cotton plants that are expressed in response to *V. dahliae* infection. Genetic markers identified in this work will be employed to assist in the breeding of cotton varieties with enhanced levels of tolerance to Verticillium wilt.

5. Acknowledgements.

We wish to thank Mr. Peter Reid and Dr. Greg Constable for providing seeds and pedigrees of cotton cultivars; and Drs. Stephen Allen and Joe Kochman for supplying *V. dahliae* infected cotton plants from N.S.W. and Queensland, respectively. This work was supported by Project Grants from the Cotton Research and Development Corporation and the Cooperative Research Centre for Sustainable Cotton Production.