The Development of Molecular Diagnostics for *Fusarium oxysporum* f.sp. *vasinfectum* in Australia

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1. INTRODUCTION

This review was commissioned by the Cotton Research and Development Corporation and the Australian Cotton Cooperative Research Centre "to provide ..... an independent assessment of the progress being made in each laboratory on molecular diagnostics for Fov detection".

The specific terms of reference were:

- A plain English summary of the aims and methodology of each laboratory
- An assessment of the scientific merit or otherwise of the methodology
- An indication of progress in relation to the aims of each research project
- If it is found that progress is not likely to meet stated aims, how might this be addressed?
- Is there a need or scope for better collaboration between laboratories
- If progress appears to be satisfactory, is there a need for a coordinated program to assist with validation?

The three identified "laboratory" or research programs were those of

Dr S Bentley
CRC for Tropical Plant Protection
University of Queensland

Dr B Lyon
Department of Biological Sciences
University of Sydney

Dr S Putcha
NSW Agriculture
Currently located at CAMBIA, Canberra

2. DNA DIAGNOSTICS

DNA based diagnostics has become increasingly utilised in the agriculture sector both in the diagnosis of pathogens and pests as well as in molecular marker assisted breeding. Most DNA diagnostic tests for plant pathogens are based on specific amplification of the genomic material (either DNA or RNA) from the pathogen using the polymerase chain reaction or PCR and the subsequent detection of the amplified product. The simplest method of detection of the amplified product is agarose or polyacrylamide gel electrophoresis. More sophisticated platforms have been developed particularly for the detection of multiple pathogens most of which are based on arrays.

The most important characteristics of a diagnostic protocol are that the protocol is both sensitive and specific. Thus, the major reasons that PCR diagnostics have become popular for pathogen detection are that very small concentrations of the target organism can be detected and the specificity can be varied from highly specific to broad. However, there are a number of disadvantages that must be overcome for a PCR based diagnostic test to be sufficiently robust for it to be used in a clinical environment. The disadvantage of PCR is that it is prone to both false positive and false negative results.

False positives usually result from (a) poor primer design or amplification conditions such that organisms other than the target organism are amplified or (b) contamination of either the original sample or reagents involved in the procedure. False negatives usually result from (a) poor primer design or amplification conditions in that there are variants within the population of the target organism which are not amplified, (b) poor extraction of DNA from the sample material, ie insufficient DNA is extracted or (c) contaminants in the DNA sample that inhibit the amplification.

With regard to the diagnosis of Fov, the consequences of false positives and negatives would be:

- Incorrect or inaccurate determination of the incidence and distribution of Fov
- Incorrect or inaccurate assessment of the resistance reaction of different cultivars
- Incorrect or inaccurate assessment of the efficacy of control measures.

Thus, PCR based diagnostic are highly effective and valuable diagnostic tools but must be carefully developed and employed to minimise the risk of false positives and negatives.

There are three components in this development and employment: (i) primer design and amplification conditions, (ii) DNA extraction and preparation and (iii) the laboratory environment.

2.1 **Primer design and amplification conditions**: this is the most important component as poor primer design cannot be overcome. Primer design requires a detailed knowledge of the target organism, in this case, *Fusarium oxysporum* f.sp. *vasinfectum*.

This knowledge includes:

- a biological definition of the target organism
- epidemiology of the organism
- molecular genetic variation of the organism

Ideally, primers should be designed such that:

- they amplify DNA from the target organism including all known variants of the target organism
- they do not amplify DNA from any other organism
- they are robust; ie, minor variations in amplification conditions will not result in false positives or negatives

2.1.1 Primer design for Fov

2.1.1a Biological definition of Fov. Fov is defined as a *Fusarium oxysporum* that is pathogenic to cotton, that is, it invades cotton and causes disease in susceptible cultivars. This is important as it is possible for other *Fusarium oxysporum* isolates to invade the cotton vascular tissue without causing disease.

Thus, for an isolate to be defined as Fov, it must be pathogenic to cotton. This can only be confirmed by pathogenicity tests.

2.1.1b Epidemiology of Fov

Fov is a soil inhabiting fungus which is pathogenic to cotton as well as species in a number of other families. It is widely distributed and is present in most cotton growing regions of the World. It is spread through the movement of infested soil and water or infected seed. It can survive for long periods in soil and plant debris.

2.1.1c Molecular genetic variation of Fov

Bentley *et al* (pers comm) have compared isolates of Fov from Australia with Fov isolates from a number of other regions of the World. Using DNA

fingerprinting, they have determined that there are two strains of Fov in Australia and importantly these two strains are different from isolates/strains/races from other parts of the World.

DNA fingerprinting techniques such as those used to determine that the two strains of Fov are distinct are excellent for determining genetic variability when using DNA from pure cultures of the target organism but are not suitable for repetitive diagnostics from mixed DNA sources such as infected plant material and soil. Direct amplification of known sequences is the most widely accepted method and the most commonly targeted genomic region is the ribosomal DNA regions (see Figure 1).

Ribosomal DNA (rDNA) consists of three genes, the 5.8S, 18S unit and 28S genes, two internal transcribed spacers regions, ITS1 and ITS2, and the intergenic spacer region, IGS. Molecular genetic analysis of both the ITS regions and the IGS regions are widely used to compare species within genera, genera within families as well as intraspecies variation.

Importantly, it is the ITS and IGS regions that are commonly used as the genomic regions from which primers are designed for PCR diagnostics.

The ITS regions are highly conserved and usually have little intraspecies variation. They are commonly used to differentiate between species or between genera. The IGS region is less conserved and is often used for intraspecies differentiation. However, it is often possible to design primers from the ITS regions that will differentiate within species.

The sequence of the IGS region has been used extensively to determine intraspecific variation within *Fusarium oxysporum*). However, there are no previous reports for the use of primers derived from the IGS region for PCR diagnostics of Fov. In contrast, the ITS regions have been used extensively to differentiate species within *Fusarium*) and, importantly, the only published report of PCR diagnostics for Fov is based on amplification of the ITS region. (Moricca *et al*, 1998).

The sequences of the ITS and IGS regions of the two Australian strains of Fov have neither been published nor made available in GenBank.

Finally, a range of amplification conditions need to be tested to ensure the robustness of the reaction and the specificity of the reaction.

2.2 DNA extraction and preparation

A protocol for the extraction of DNA in a condition that can be amplified is an essential component of any diagnostic procedure. This is a relatively simple task from pure cultures of a fungus but becomes increasingly more difficult and complex when extracting 'amplifiable' DNA from plants and soil.

However, there are a large number of published methods and kits available

and thus it should be possible to quickly determine appropriate methods of extraction of 'amplifiable' Fov DNA from plants and soil.

2.3. Laboratory Environment

There is a considerable difference between developing a PCR diagnostic protocol in a research environment and providing PCR diagnostics on a routine basis. The latter requires (i) both quality control and assurance programs and (ii) standard operating procedures including detailed record keeping. These are rarely implemented in a research environment.

Thus, the development of a Fov diagnostic test should include a plan for technology transfer to a plant disease "diagnostic clinic" which may be either a private or public facility.

3. THE CENTRAL ISSUE

From discussions with each of the three project leaders, it was clear that the central issue involved the selection of target sequence within Fov to be amplified. This in turn determined from where the primers would be designed.

Both Dr Lyon and Dr Putcha had chosen the ITS region probably initially based on the paper of Moricca et al (1998). However, as the ITS region is highly conserved, the resultant primer design meant that there was only a one or two base pair mismatch between the primers for Australian Fov and the equivalent sequences of the ITS regions of other *Fusarium oxysporum*. For such primers to differentially only amplify Fov, stringent amplification conditions must be imposed, as indicated by Dr Putcha. Because of the necessity for such stringent conditions, the robustness of a diagnostic based on ITS derived primers is significantly reduced.

In contrast, Dr Bentley has targeted the IGS region and, because of the greater genetic variation in this region, has been able to design primers that are specific for each of the two strains of Australian Fov. Importantly, these primers are significantly different to the equivalent IGS sequences in other *Fusarium oxysporum* and other fusaria. Thus, amplification conditions do not need to be as stringent and would be prone to fewer false positives or negatives than amplification of the ITS region.

The downside of the increased specificity offered by targeting the more variable IGS region is that if there is a third strain/ VCG of Fov in Australia it may not be amplified by the IGS primers but would be more likely to be amplified by the ITS primers of Dr Lyon and Dr Putcha.

In summary, the amplification protocol developed by Dr Bentley based on IGS primers is likely to be the more robust and definitive Fov diagnostic test with the proviso that a pair of *'Fusarium oxysporum'* specific primers are

used in parallel to detect a potential third VCG in infected cotton plants and as a positive control for extracts from soil. Dr Bentley has proposed to include such a primer set.

4. RESPONSE TO THE TERMS OF REFERENCE

Two of the laboratories, those of Dr Lyon and Dr Putcha, have not been funded to develop Fov diagnostics. Rather, they have developed Fov detection protocols as an imperative for their projects aimed at controlling Fov in the Australian cotton crop. Dr Bentley's laboratory has been specifically funded to develop Fov diagnostics.

4.1 Dr Bruce Lyon's Program

"A plain English summary of the aims and methodology:

The principal cotton research activity of this laboratory is to develop molecular markers for Fov and Verticillium resistance. An important part of this research has been to challenge a range of cotton genotypes/cultivarsunder glasshouse conditions and assess the level of infection. Initially, this involved identifying the appearance of disease symptoms in inoculated plants.

More recently, Dr Lyon and his CRC funded PhD student, Augusto Becerra, have developed a PCR test based on amplification of the ITS region to (a) detect Fov or Verticillium in glasshouse challenged cotton prior to the appearance of symptoms and (b) differentiate between Fov and Verticillium infection in glasshouse challenged cotton.

The laboratory has also used the protocol to amplify from DNA extracts of 20 plants from one cotton field near Narrabri.

"An assessment of the scientific merit or otherwise of the methodology and an indication of progress in relation to the aims of the project".

Dr Lyon's laboratory essentially developed PCR detection of Fov and Verticillium as a tool to improve the efficiency of his project to develop molecular markers for disease resistance. In this application, the tool is entirely appropriate. At the time of development, no such detection methods were available for Australian Fov.

In a "side project" apparently not funded by the cotton industry, Dr Lyon's laboratory used the method to amplify DNA from 20 cotton plants from a field near Narrabri. One of the sequences amplified using ITS primers was a perfect match with Fov. This result has caused considerable concern as no Fusarium wilt of cotton has been reported from this field. This needs to be resolved. Most importantly, this isolate should be tested for pathogenicity as if it does not cause disease in susceptible cotton cultivars then it cannot be classified as Fov. It is entirely possibly that this is a non-pathogenic *Fusarium*

oxysporum. It would also be very useful for the IGS region of this isolate to be sequenced and compared with those of the two strains of Australian Fov.

This is an example of relatively incomplete information published in a magazine without scientific refereeing causing unnecessary concern to industry.

"If it is found that progress is not likely to meet stated aims, how might this be addressed".

The methodology developed by Dr Lyon's laboratory has been used as part of the funded research to develop the molecular markers and is a powerful tool for this purpose. **However, if Dr Lyon wished to develop his methodology as the 'industry standard' for Fov diagnostics, there would need to be a great deal of further validation to be done. This would include:**

- **an assessment of the stringency of amplification conditions required to obtain the necessary specificity to amplify only Fov**
- **large scale testing of field collected plant material**
- **development of the methodology for detection of Fov in soil**

4.2 Dr Subbu Putcha's Program

"A plain English summary of the aims and methodology"

The principal cotton research activity of this program is to investigate the control of cotton seedling diseases and vascular wilts with microorganisms. An important component of this project is the ability to monitor a changing population of the pathogen in soil and to monitor increases or decreases in disease incidence as a consequence of various treatments. To this end, Dr Putcha began to develop DNA diagnostics for Fov in late 1998 or early 1999. At that time as now, there was no published information on the ITS or IGS sequences of Australian Fov strains.

Dr Putcha tested the primers used by Moricca et al (1998) but found the results to be too variable. Consequently, he has sequenced both the ITS and IGS regions of Australian Fov. He has used both IGS and ITS primers, including a combination of both but now routinely uses two ITS primers of his design.

"An assessment of the scientific merit or otherwise of the methodology and an indication of progress in relation to the aims of the project".

It is clear that Dr Putcha has developed a very useful methodology for detecting Fov. He has tested his protocol against a wide range of fungi including other

Fusarium oxysporum and fusaria and over 100 plant samples. He is confident of his methodology for plant samples.

He has also used his methodology with soil samples. He has developed protocols for extraction of 'amplifiable' DNA from soil but feels his soil testing application needs further development.

The major concern is that stringent conditions for amplification must be used as the four closest non-Fov *Fusarium oxysporum* differ by only one nucleotide from Dr Putcha's ITS primers for Fov. The use of such primers could easily result in false positives particularly from soil where it is reasonable to expect that different *Fusarium oxysporum* would be encountered.

"If it is found that progress is not likely to meet stated aims, how might this be addressed".

Dr Putcha has made very good progress in developing a methodology based on ITS primers designed from the sequence of Australian Fov ITS regions and has tested this against other Fusaria and *Fusarium oxysporum*. Further, he has developed both plant and soil DNA extraction protocols.

However, as with Dr Lyon's methodology also based on ITS primers, there would be a concern with false positives particularly from soil samples. **Again, if this approach were to be used as the 'industry standard' for Fov diagnostics, there would have to be extensive validation against a range of plant and soil samples.**

4.3 Dr Suzie Bentley's Program

"A plain English summary of the aims and methodology"

This is the only project specifically funded to develop Fov diagnostics.

Dr Bentley's group have taken a structured and planned approach which involved, firstly, the identification by DNA fingerprinting of two strains of Fov in Australia, the sequencing of the ITS and IGS regions of both strains and comparison of those sequences against all others in GenBank. From this information, primers were designed against the IGS region such that amplification with the two sets of primers would differentiatethe two Australian strains of Fov and not amplify DNA from any other organisms. These primer sets have been tested against a large panel of DNAs extracted from a range of fungi including other *Fusarium oxysporum* and other fusaria.

Testing with DNA extracted from plants and soil is yet to commence.

"An assessment of the scientific merit or otherwise of the methodology and an indication of progress in relation to the aims of the project".

From sequence data available from the ITS and IGS regions of Fusarium spp., it is the reviewer's opinion that primers designed from the IGS region of Australian Fov are considerably more likely to provide the greatest specificity (least propensity for false positives), particularly in a diagnostic laboratory setting.

However, it should be noted that while the two primers sets have performed as expected with DNA extracted from pure cultures of a wide range of fungi, testing against field plant and soil samples is yet to commence. This is expected to begin towards the end of 2000 which is in line with the predicted progress of the project.

Perhaps the greatest risk of using such highly specific primer sets is the potential to "miss" a variant of Fov that has not been identified as yet - the hypothetical third strain which may or may not exist. The Bentley group are now designing *Fusarium oxysporum* specific primers which, when used in parallel with the Fov IGS primer sets, should identify suspect *Fusarium oxysporum* isolates in plant samples which would lead to further investigation. These primers, however, will not identify "the third strain" in soil samples because of the very common occurrence of *Fusarium oxysporum* in soils.

"If it is found that progress is not likely to meet the stated aims, how might this be addressed".

There is no reason to believe that this project will not meet the stated aims; however, the next 12 months will be definitive as it is during this period that the IGS primer sets will be tested with DNA extracted from soil and plant samples. **Thus, reporting on these milestones should be closely monitored and a comprehensive report should be scientifically reviewed prior to the acceptance of this methodology as the industry standard and transfer of the technology to a plant disease diagnostics laboratory.**

4.4 All three programs

"Is there a need or scope for better collaboration between laboratories".

From my discussions, it would appear that there is reasonable communication between Dr Lyon and Dr Putcha (both using ITS primers). However, there appears to have been less positive interaction with Dr Bentley's group. This is almost certainly a consequence of the different approaches to primer design. This is unfortunate as all three are very capable scientists and each making an important contribution to the cotton industry.

It is my assessment that Dr Bentley's approach will yield a robust, sensitive and specific diagnostic test for Fov in Australia and assuming that progress continues as predicted will become the accepted method for the cotton industry.

It is currently unlikely, however, that Drs Putcha and Lyon will adopt this methodology because (i) it will require time and effort to establish a different methodology in their respective laboratories and (ii) both assume that once the method has been transferred to a diagnostic service that there will be a financial cost involved in accessing the method.

To overcome at least part of this impediment, it is my recommendation that which ever methodology is accepted and implemented for the diagnosis of Fov for the Australian cotton industry, this method complete with protocols and primers sequences be made freely available for research purposes only, to research programs funded at least in part by the Australian cotton industry.

"If progress appears to be satisfactory, is there a need for a coordinated program to assist with validation".
The aims of the three programs reviewed are very different and, in my opinion, progress in each has been satisfactory. Two of the programs were not funded to develop Fov diagnostics but have developed detection methods as tools to increase the efficiency of the programs. This is to be commended.

The program funded specifically to develop Fov diagnostics has also progressed more than satisfactorily and should be supported through validation of the methodology which should be completed my mid 2001. The results of this validation period should be scientifically reviewed prior to acceptance by industry and technology transfer.

As neither Dr Lyon's nor Dr Putcha's programs have been funded to develop Fov diagnostics and that Dr Bentley's funded project is progressing more than satisfactorily, I recommend that there is no necessity to conduct extensive validation experiments unless the methodology developed by Dr Bentley proves either ineffective or inaccurate.

4.5 **The overall term of reference** for this review was: "to provide the corporation and the ACCRC with an independent assessment of the progress being made in each laboratory on molecular diagnostics for Fov detection".

In response, I have provided, in Table 1, a summary of the approaches, advantages and progress of each of the three laboratories. It is my considered opinion that the project funded specifically to develop Fov diagnostics is most likely to deliver to industry a robust, sensitive and specific Fov diagnostic test because:

- the primer sets have been rationally designed from Fov sequences and sequence comparisons and are targeted to a region with greater intraspecific variation

- the test can differentiate between the two strains of Australian Fov

- the test is less likely to yield false positive results particularly from soil samples

- the program has a clear strategy to transfer the technology to a diagnostics laboratory greatly increasing the likelihood that the test will become generally available.

It should however be recognised that both Dr Lyon and Dr Putcha have made significant contributions to Fov detection in Australia; both developed protocols which enhanced the progress of their projects and, in the event that Dr Bentley's methodology cannot be validated, provide a potentially viable alternative. It must be remembered that if neither had developed Fov detection methodology, their programs today would not have access to Fov DNA diagnostics.

	Target sequence	Differentiate Australian strains	Propensity for false positives	Testing against other fungi (pure cultivars)	Testing with plant extracts	Testing with soil extracts	Implementation strategy
Lyon	ITS	No	+++	++	+	nc	No
Putcha	ITS	No	+++	+++	++	+	Possible
Bentley	IGS	Yes	+	+++	nc	nc	Clear

nc = not commenced