Improved PCR-based detection of Sigatoka disease and black leaf streak disease in Australian banana crops

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Abstract

Accurate diagnosis of black leaf streak disease is often complicated by the presence of other fungal pathogens and in particular by the morphological similarity of the related species Mycosphaerella musicola, the causal agent of Sigatoka disease. In addition, high rainfall often washes away fungal structures making microscopic identification difficult. Starting in 1998, the Queensland Department of Primary Industries has been using molecular methods to help diagnose black leaf streak disease. A polymerase chain reaction (PCR) assay was used, but the protocol was found to lack specificity when applied to Australian isolates of the fungi. In July 2000, a project aimed at improving the sensitivity and specificity of the PCR as well as streamlining the assay was initiated. Various components of the PCR test were targeted for improvement. Homogenization of banana leaf tissue has eliminated possible cross-contamination while tripling batch throughput. An improved DNA extraction method produces cleaner DNA in less than half the time of the prior extraction method. Flexibility and sensitivity of the PCR has been improved by introducing a new enzyme while the new format PCR thermal cyclers have increased sample throughput. Importantly, specificity has been enhanced with the design of new diagnostic primers. These changes produce a definitive result during the first PCR in more than 98% of samples while increasing daily throughput more than eight-fold.

Resumen - Mejoramiento de la detección de la Sigatoka negra y Sigatoka amarilla basada en PCR en los cultivos bananeros de Australia

A menudo el diagnóstico preciso de la Sigatoka negra es complicado debido a la presencia de otros patógenos fungosos y en particular por la similitud morfológica de la especie relacionada Mycosphaerella musicola, agente causal de la Sigatoka amarilla. En adición, fuertes precipitaciones a menudo se llevan las estructuras fungosas dificultando la identificación microscópica. El QDPI ha estado utilizando métodos moleculares para confirmar el diagnóstico de la Sigatoka negra desde 1998. Se utilizó un ensayo de la reacción en cadena de polimerasa (PCR), sin embargo se descubrió que al protocolo le faltaba la especificidad al aplicarlo a los aislados australianos de los hongos. En julio de 2000, se inició un proyecto dirigido a mejorar la sensibilidad y especificidad del PCR así como la modernización del ensayo. Se seleccionaron varios componentes del examen

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PCR para ser mejorados. La homogenización del tejido foliar del banano con la ayuda de una micromano de mortero plástica dentro del tubo ha eliminado una posible contaminación cruzada al triplicar el rendimiento de los lotes. Un método mejorado de extracción de ADN produce un ADN más limpio en menos de la mitad del tiempo, que el método de extracción anterior. La flexibilidad y sensibilidad de PCR fueron mejoradas introduciendo una nueva enzima, mientras que nuevos variadores térmicos para el formateo de PCR han aumentado el rendimiento de las muestras. La especificidad de PCR ha sido mejorada a través del diseño de nuevos iniciadores de diagnóstico. Combinadas, estas mejoras producen un resultado definitivo durante el primer ensayo de PCR en más del 98% de las muestras, mientras que el rendimiento diario de la muestra es 8 veces mayor.

Résumé - Détection améliorée basée sur la PCR de la maladie de Sigatoka et de la maladie des raies noires dans les plantations de bananes en Australie

Le diagnostic exact de la maladie des raies noires est souvent rendu plus difficile par la présence d'autres pathogènes fongiques, et en particulier par la similarité morphologique d'une espèce voisine Mycosphaerella musicola, l'agent causal de la maladie de Sigatoka. De plus, de fortes précipitations éliminent souvent des structures fongiques ce qui rend l'identification au microscope difficile. En 1998, le Queensland Department of Primary Industries (QDPI) a commencé à utiliser des méthodes moléculaires afin de mieux identifier la maladie des raies noires. Un essai basé sur la réaction en chaîne par polymérase (PCR, polymerase chain reaction) a été utilisé mais ce protocole était peu spécifique quant aux isolats australiens du champignon. En juillet 2000, une étude a été initiée afin d'augmenter la sensibilité et la spécificité de la PCR et pour rationaliser le protocole. Divers composants du test PCR ont été ciblés afin d'être améliorés. L'homogénéisation des tissus de la feuille de banane a permis d'éliminer les contaminations extérieures tout en triplant le débit. Une méthode améliorée d'extraction de l'ADN permet d'obtenir un ADN plus pur en deux fois moins de temps. La flexibilité et la sensibilité de la PCR ont été améliorées grâce à l'utilisation d'une nouvelle enzyme. De plus, le nouveau format des thermocylereurs PCR a permis d'accroître le débit. Il est important de noter que la spécificité a été mise en valeur par la conception de nouvelles amorces diagnostiques. Ces changements produisent un résultat définitif dans la première PCR dans plus de 98% des cas et multiplient par huit le nombre d'échantillons traités par jour.

The Tully 2001 black leaf streak disease outbreak

The value of the Australian banana industry is estimated to be A$357 million (US$193 million) per year. In 2000, nearly 250 000 tonnes of bananas were produced by 100 growers in Australia. All Australian bananas are produced for consumption locally and 85% are of the ‘Cavendish’ variety. The majority of bananas are grown in northern Queensland, with 67% of the crop concentrated in Tully, Cairns and Innisfail (Figure 1).

In April 2001, the Australian banana industry suffered a potentially devastating outbreak of black leaf streak disease (caused by Mycosphaerella fijiensis) in Tully (Figure 1). This is the pathogen’s first incursion in a major commercial region in Australia; failure to control the pathogen would have far reaching effects on the industry.

The Queensland Department of Primary Industries (QDPI) has had considerable success eradicating previous outbreaks of black leaf streak disease by plant destruction and replacement with resistant varieties. Since the initial discovery of black leaf streak disease in 1981 at Bamaga, an Aboriginal community located 40km
from the tip of the Cape York Peninsula, black leaf streak disease has been detected and eradicated eight times in far north Queensland. This ninth outbreak was in Tully where crops are estimated to be worth A$119 million per year (US$64 million).

Diagnosis of black leaf streak disease in Australia

Banana crops are routinely surveyed for black leaf streak disease by QDPI scientists at the Centre for Tropical Agriculture, Mareeba. Accurate diagnosis of black leaf streak disease is complicated by the morphological similarity of *M. fijiensis* to a related species *M. musicola*, which causes Sigatoka disease. Usually, experienced plant pathologists distinguish the two diseases by the development of symptoms and microscopical features of the fungi. In Tully, conidia were absent because of prolonged rainfall, and identification of morphological characters was not possible. Therefore, molecular methods were used for diagnosis.

The QDPI has used the polymerase chain reaction (PCR) to confirm diagnoses of *Mycosphaerella* leaf spot diseases since 1998 (Johanson, 1997). Approximately 10% of laboratory samples required confirmation by PCR. However, the method was slow and lacked specificity to some Australian and Torres Strait Island isolates of the fungi. The lack of specificity was possibly due to the high variability among the Southeast Asian populations of the pathogen. Populations of *M. fijiensis* from the Torres Strait were found to differ from those of the Pacific (Hayden, 2001). However, the Torres Strait populations and Pacific populations were found to be related to those from Papua New Guinea where there is a considerable diversity (Carlier, pers. comm.). In the original
study by Johanson et al. (1994) isolates from the Torres Strait were not included and it is possible that these isolates could be the source of the variability not detected by the original PCR primers.

In July 2000, a project between QDPI and the Cooperative Research Centre for Tropical Plant Protection (CRCTPP) was initiated with the aim of improving the specificity of the diagnostic procedure and increasing throughput in readiness for outbreaks of the disease1. Aspects of the PCR diagnostic procedure that were targeted for improvement included sample excision, homogenisation of banana leaf tissue, DNA extraction, PCR protocol, PCR primer design and equipment.

Flame sterilised cork-borers 4 mm in diameter have replaced scalpels for the removal of suspect lesions from banana leaves. The method is quick and simple and there is no cross-contamination between samples. Plastic micropestles have replaced ceramic mortar and pestles for homogenising leaf tissue. Micropestles have reduced the potential for cross-contamination between samples, eliminated transfer from mortar to tube, and have tripled the throughput. The rapid cetyltrimethyl ammonium bromide (CTAB) DNA extraction method (Stewart and Via, 1993) was adopted. It produces cleaner DNA in half the time.

New PCR primer sequences specific to *M. musicola* and *M. fijiensis*, and a modification of published ribosomal gene primer sequences (White et al., 1990) to improve specificity to increase duplex stability of the primers with the target DNA (Rychlik, 1993), has improved specificity of the PCR assay. A size difference was also included in the PCR assay with the specific primer for *M. fijiensis* designed to the ITS1 and the specific primer for *M. musicola* designed to the ITS2 (Figure 2). Flexibility and sensitivity was improved by introducing the hot-start enzyme, TaqGold DNA polymerase (PE Biosystems). TaqGold DNA polymerase requires heat-activation before amplification can proceed. Therefore, non-specific amplification products are reduced and reactions can be left at 4°C until the addition of template. New equipment at the Centre for Tropical Agriculture has also improved throughput of assays. New format PCR thermal cyclers have increased tube capacity from 30 to 192 per run, and new electrophoresis equipment can analyse 52 samples for Sigatoka disease and black leaf streak disease at the same time.

The new methods produce a high quality DNA preparation and provide a definitive result during the first PCR in more than 98% samples. In addition, extraction time is more than halved and daily throughput increased by more than eight-fold.

### Application of new molecular test

Use of the new methods in April 2001 coincided with Australia’s most severe outbreak of black leaf streak disease. This was the first outbreak in a commercial growing area; previous outbreaks had been further north and in places where containment was easy. Fungal structures were absent on banana samples because of high rainfall at Tully. Therefore, diagnosis of up to 50% of samples was confirmed using the PCR assay. The PCR assay provided the Australian government and

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1 The project is co-managed by Ron Peterson (Principal Plant Pathologist, Mareeba QDPI) and Juliane Henderson (Research Officer, CRCTPP).
banana industry the confidence to start a A$20 million (US$10.8 million) surveillance and eradication plan in Tully, and more than 2500 PCR tests have been done.

The CRCTPP and QDPI continue to monitor and improve the PCR diagnostic. Thus, scientists from the CRCTPP improved homogenization of banana tissue by the use of glass beads shaken at high speeds. The commercially available “FastPrep” Instrument (Q-Biogene) processes 12 samples in 45 seconds and eliminates cross-contamination between samples by single-use O-ring tubes. Use of the method at the Centre for Tropical Agriculture is dependant on funds. Opportunities to automate and improve specificity and sensitivity of the assay are being studied as part of the CRCTPP’s plan to use new technology. Development of a real-time PCR assay to detect and differentiate *M. fijiensis*, *M. musicola* and *M. eumusae* is in progress. A fluorescent PCR format increases sensitivity and specificity, reduces cross-contamination, and increases throughput because post-PCR processing is not required.

To ensure the robustness of the PCR diagnostic and to facilitate development of new diagnostic assays for *Mycosphaerella* leaf spot diseases in Australia, the sequence variability in Australasian isolates of *M. musicola* and *M. fijiensis* will be studied. First, the region incorporating the ITS1, 5.8S ribosomal gene and ITS2 will be cloned and sequenced from *Mycosphaerella* isolates from Australia, the Torres Strait Islands and Fiji. In collaboration with other groups studying sequences pertaining to the disease, we will compare our database with overseas isolates. If further sequence information is required, other conserved fungal genes, e.g. β-tubulin, histone-4, glyceraldehyde-3-phosphate, will be investigated. The information from this study will help us to understand how the 2001 Australian outbreak arose, e.g. whether from one or several sources.

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1 Drs Elizabeth Aitken (Department of Botany, University of Queensland) and Juliane Henderson will use joint funding from the Australian Banana Industry Protection Board (BIPB) and Horticulture Australia Limited (HAL) to investigate sequence variability of *Mycosphaerella* causing leaf spot diseases on banana.
The status of black leaf streak disease in Australia is yet to be confirmed and the future application of diagnostic tests is uncertain. The method could be used to maintain Australia’s disease-free status, as far as black leaf streak disease is concerned, or to monitor pathogen populations for control measures should the disease become endemic. Either would ensure that the best diagnostic assay is available to the Australian banana industry.

References


Johanson A. 1997. Detection of Sigatoka Leaf Spot Pathogens of Banana by the Polymerase Chain Reaction. Natural Resources Institute, Chatham, UK.

