

Easy steps for using the Smart Cycler®

drive to the field



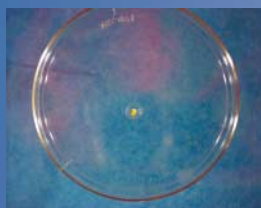
remove diseased tissue sample



drive to the lab



incubate tissue in 50 µL water for 20 min.



add 1 µL sample to reaction tube



run PCR for 20 min. to get results



Elapsed time: 60 minutes*

*except in traffic jams

On-site one hour PCR diagnosis of bacterial diseases

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Classical diagnosis of a bacterial disease normally requires 3–4 days to isolate and clone the suspect pathogen and another 5–7 days to confirm the organism's identity by pathogenicity tests. More rapid serological tests are available, but they often lack the sensitivity or result in false positives. Classical PCR tests are very sensitive but usually require 2–3 days. We describe a real-time TaqMan®-based PCR protocol adapted to a field-portable Smart Cycler® (Cepheid, Sunnyvale, CA) for rapid same-day diagnosis of watermelon fruit blotch (WFB), caused by *Acidovorax avenae* subsp. *citrulli*. Major advantages of the Smart Cycler® include portability and rapid cycling times. In direct comparisons with Applied Biosystems Prism™ 7700 Sequence Detection System, we found no differences in specificity and little in sensitivity. Both systems were able to detect 100 fg DNA. Using a Smart Cycler®, WFB can be diagnosed by direct PCR in less than 1 h after sampling using 1 µL of washings of diseased tissue soaked for 20 min in water in a microfuge tube.

Introduction

Plant disease diagnosis has improved greatly with the introduction of PCR (7). Classical PCR assays are available for several plant pathogens (3) and primers are available for the identification and detection of most plant pathogenic bacteria (9). Because classical PCR techniques require time consuming Southern blot hybridization for confirmation, field diagnosticians have shown little interest in using PCR for routine diagnosis. The introduction of real-time PCR, which is based on hybridization to a fluorescent probe sequence, has led to the development of protocols for detection of several plant pathogens (2, 6, 10). Such techniques are more sensitive than classical PCR and greatly reduce the time needed for diagnosis since there is no need for agarose gels and Southern blots. However, real-time PCR generally requires expensive lab-based equipment. In response to a disease quarantine issue involving shipment of perishable goods or the deliberate release of a pathogen, time becomes an issue of critical importance. To demonstrate the feasibility of rapid on-site diagnosis of a plant disease in one hour or less, we developed a real-time PCR protocol using the portable Smart Cycler® TD [Cepheid, Sunnyvale, CA (3)] for the

destructive watermelon fruit blotch disease caused by the bacterium, *Acidovorax avenae* subsp. *citrulli*. Watermelon fruit blotch causes severe losses in watermelon production worldwide. Symptoms of the disease in seedlings grown under greenhouse or field conditions often consist of large black lesions without any water-soaking. This makes presumptive diagnosis based solely upon symptom etiology very difficult. Isolation of bacteria and pathogenicity tests are generally required for diagnosis but take 10–14 days.

Materials and Methods

PCR protocol. The *A. avenae* subsp. *citrulli* specific PCR primers, AacF3 and AacR2 and FAM-labeled probe AacP2 (8), were used in real-time PCR with the following cycling conditions with the Smart Cycler®: 95 °C for 30 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The assays were performed in a total volume of 25 µL containing the following reagents and volumes (µL): 10x PCR buffer (2.50), 25 mM MgCl₂ (5.00), 1.25 mM dNTPs (4.00), 25 µM primer AacF3 (1.00), 25 µM primer AacR2 (1.00), 10 µM probe AacP2 (1.00), 5U/µL AmpliTaq® DNA polymerase (0.25), 5x additive reagent containing BSA at 1 mg/mL, Trehalose at 750 nM, and Tween-20 at 1% v/v (Cepheid, 5.00); sterile MBG water (4.25), sample (1.00). The cycle threshold (Ct) values were determined and samples with a Ct value of 38 or less were considered positive. Ct value is defined as the PCR cycle number at which time the signal (fluorescence) of the probe rises above background. The earlier the Ct value the better the PCR performance.

Sensitivity of detection of pure cultures and extracted DNA.

For pure cultures, an overnight suspension of *A. avenae* subsp. *citrulli* was adjusted to 0.1 at 600 nm and diluted to 1×10⁻⁸. One microliter aliquots of the 10⁻⁵ to 10⁻⁸ dilutions were run for 40 cycles in duplicate using an ABI 7700 and a Smart Cycler®. For viable cell counts in tissue samples, 100 µL of each dilution were plated onto each of five plates of YDC agar and incubated at 36 °C for 2 days. DNA was extracted from cells using standard phenol/chloroform methods (1) and quantitated using a SmartSpec® 3000 (BioRad, Oakland, CA).

Preparation of plant samples.

Watermelon seedlings at the 1st true leaf-stage were injected with a suspension containing 1×10^6 cfu/mL of *A. avenae* subsp. *citrulli* into the cotyledon and stem. After 7–10 days incubation in a lighted dew chamber, plants with brown-black lesions on the cotyledon (Fig. 1A) or brown linear lesions on the stem (Fig. 1B) were collected. Discs of tissue were removed from the edges of the cotyledon lesions and 2–5 mm tissue sections were cut from the upper and lower margins of the stem lesions (see arrow) with a scalpel. The tissue was soaked in 50–100 μ L sterile water in a microfuge tube for 20 min at room temperature, and 1 μ L was immediately used for PCR without DNA extraction.

Results

Sensitivity. The sensitivity of the Cepheid Smart Cycler[®] was comparable to the ABI 7700[®]; both instruments were able to detect at least 100 fg and as few as 10,000 cells of *A.*



Fig 1A: lesions on the cotyledon



Fig 1B: lesions on the stem

avenae subsp. *citrulli* per mL (10 cells/ μ L) of tissue sample extract. Tissue samples containing a mean of 1.1×10^4 cfu/mL resulted in Ct values of 32.4 and 34.5 for the Smart Cycler[®] and 7700[®], respectively. Viable cell counts of *A. avenae* subsp. *citrulli* in stem soaks ranged from 10^4 to 10^6 cfu/mL.

Sampling and detection. PCR tests were completed in less than 1 h after receiving infected samples. All stem and leaf samples tested were positive whereas all samples from non-inoculated control plants were negative. Typical Ct values ranged from 24.16 to 26.25 (Fig 2).

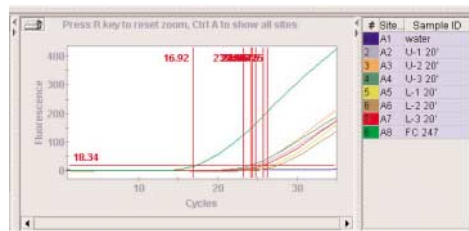


Fig. 2: Real-time diagnosis of WFB

Discussion

By using the fast cycling Smart Cycler[®] and sampling without extracting DNA, we were able to detect WFB in seedlings in less than one hour after obtaining samples. Such rapid diagnosis is critical to the successful development of a pest management control program aimed at early detection to prevent spread of the pathogen. The extremely fast cycling of the Smart Cycler[®] is due in part to the unique design of the PCR reaction tube and optical mechanism. No evidence of PCR inhibition was observed as a result of our sampling protocol. Unlike the protocol developed for rapid detection of *Neisseria gonorrhoeae* (5), our protocol for detection of the watermelon fruit blotch organism does not require extraction of DNA and is therefore much faster.

Literature

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Summary

- Demonstrated for the first time diagnosis of a plant disease by PCR in less than 1 hour.
- Developed simple sampling procedure by soaking tissue 20 minutes in 50 μ L water.
- Conducted PCR directly from diseased samples — no DNA extraction needed.
- High sensitivity observed; 100 fg DNA or 10 viable cells/ μ L were detected.
- The real-time PCR assay was completed in 20 minutes using the Smart Cycler[®].
- Using a portable Smart Cycler[®], PCR is performed easily in a greenhouse or field.