Real-time fluorescent PCR detection of fungal plant pathogens using the Smart Cycler

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Objective

Evaluate real-time PCR assays for Karnal bunt and soybean rust using the Smart Cycler (Cepheid, Inc., Sunnyvale, CA).

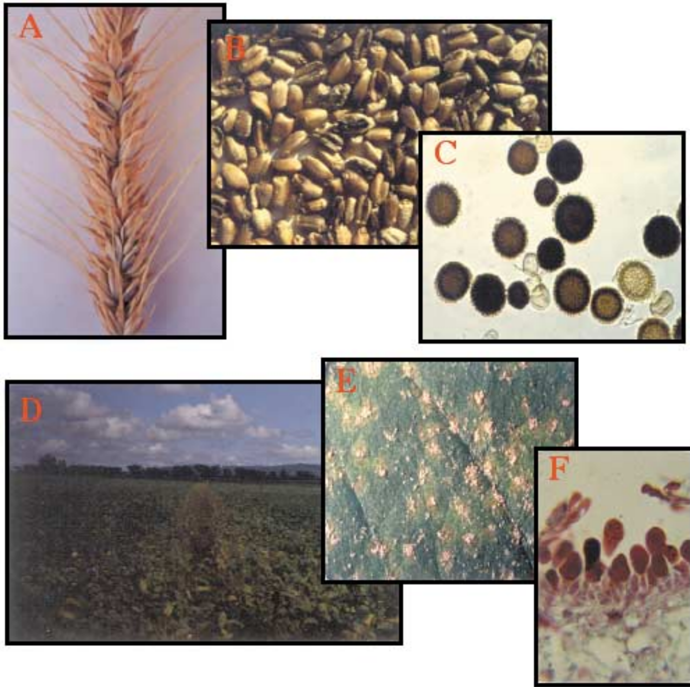

Figure 1: Karnal bunt: a) infected wheat plant, b) bunted kernels, and c) teliospores. Soybean rust: d) infected soybean field in Zimbabwe, e) urediniospores on soybean leaf, and f) cross-section through uredium (970 x).

Figure 2: The portable Smart Cycler TD. Real-time PCR assays can be conducted at remote/field locations using the Smart Cycler. The Smart Cycler processing block has 16 independently-controlled reaction sites (I-CORE modules) allowing different cycling parameters to be run simultaneously.

Introduction

Plant diseases greatly reduce agricultural productivity in the United States, and new pathogens are continuing to emerge that create new disease problems requiring novel control measures. Accurate and timely diagnoses of plant disease are extremely important so that appropriate controls can be implemented quickly. Disease symptoms often aid with making decisions, but a definitive diagnosis requires unambiguous pathogen identification. A major mission of the USDA/ARS Foreign Disease-Weed Science Research Unit is to examine new technologies and develop novel methods for the detection and identification of exotic plant pathogens that are deemed significant threats to United States agriculture. Karnal bunt of wheat and rust on soybeans are two fungal diseases that are currently under study.

- Karnal bunt is an important disease of international trade, and in 1996 the pathogen, *Tilletia indica Mitra*, was found for the first time in the United States in areas of Arizona and California. Another closely related smut pathogen that infects ryegrass, *T. walkeri*, produces teliospores that are morphologically similar and difficult to distinguish from teliospores of *T. indica*.
- Soybean rust is a serious disease on soybeans that results in yield reductions due to premature defoliation of infected leaves. Two different *Phakopsora* species cause soybean rust. The soybean rust pathogen in Asia and Australia is *P. pachyrhizi Sydow*, while *P. meibomiae Arthur* is the tropical Latin American or New World rust species. Soybean rust is not currently found in the continental United States; however, serious yield losses due to soybean rust are likely to occur if the pathogen gained entry into the major soybean production regions of the United States.
- Previously, real-time PCR assays that are capable of distinguishing between *T. indica* and *T. walkeri* (1) and between *P. pachyrhizi* and *P. meibomiae* (2) were developed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA).

Materials & Methods

Fungal Isolates

Tilletia isolates were obtained from India, Mexico, and the United States.

Phakopsora isolates were collected from Australia, Brazil, Hawaii, India, Philippines, Puerto Rico, Taiwan, and Thailand. All fungal isolates were maintained at the USDA-ARS Biological Containment Facility at Ft. Detrick under APHIS permit. *Tilletia* isolates were grown on potato dextrose agar (PDA). Rust isolates were propagated by inoculation onto the soybean cultivar "Williams" (*P. pachyrhizi* isolates) or onto red kidney bean (*P. meibomiae*).

DNA Extraction

Mycelia was harvested from sporidial grown potato dextrose broth cultures of the *Tilletia* isolates, and the tissue was ground in 1.5-mL microcentrifuge tubes using a plastic pestle attached to a power drill. DNA was purified using a Puregene DNA isolation kit (Gentra Systems) according to the manufacturer's directions. *Phakopsora* urediniospores were ground in 100 µL of extraction buffer (89 mM Tris-HCl (pH 8.0), 45 mM boric acid, 0.05 mM EDTA, and 1.0% (vol/vol) β-mercaptoethanol) in microcentrifuge tubes using a plastic pestle attached to a power drill. The samples were incubated at 75 °C for 15 minutes and were centrifuged for 10 minutes at 16,000 x g to pellet debris. The supernatant was transferred to new tubes and stored at –20 °C as DNA extracts.

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Pme  GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAAT    56
Ppa  GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTAAT    56

Pme  AAAAAGCTTAAAAAAGAGTGCACTTAATTGTGGCTCTAAACACAAAACTTTTTATA    112
Ppa  AAAAAGCT----AAAGAGTGCACTTTATTGTGGCTC- AAAACTAAACTTTTTA-A    105

Pme  TAAACCCATTTAAACTGGCTCAGCTACTTCATTGA--AGTT-TTTGGGCAAATCAC    165
Ppa  TAAACCCATTT-AATTGGCTCA-----TTGATTGATAAGATCTTTGGGC-AATGGT    154

Pme  AGCTTTGAAAAAAGTTGCAAATCATCTATTATATCATTGTCATTTTTTATTTTATT    221
Ppa  AGCTTTGAAAAAAGCTGCAACCCACCTATTA-ATCATAATCTTTTTTTTTT-----    204

Pme  AAATAAC-CAAAGTCACAAAGAATGTTTTATAAATTTTAAAAAATATATATAACTT    276
Ppa  ---TAACTCAAAGTCAAATAGAATGTTTTATAAATTTAAATATATATATATAACTT    257

Pme  TTAGCAATGGATCTCTAGGCTCTCATATCGATGAAGAACACAGTGAAATGTGATAA    332
Ppa  TTAACAATGGATCTCTAGGCTCTCATATCGATGAAGAACACAGTGAAATGTGATAA    313
                     Ppm1'er ---->
Pme  TTAATGTGAATTGCAGAATTCAGTGAATCATCAAGTTTTTGAACGCACCTTGTACC    388
Ppa  TTAATGTGAATTGCAGAATTCAGTGAATCATCAAGTTTTTGAACGCACCTTGCACC    369
                     FAM probe
Pme  TTTTGGTATTCCAAAAGGTACACCTGTTTGAGTGTCATGAATTATTCTCAAC--TC    442
Ppa  TTTTGGTATTCCAAAAGGTACACCTGTTTGAGTGTCATGAAATCTTCTCAACATTA    425
                                <----  Pme2'er
Pme  TTTCTTTATTTACTTAAAG--AAAAGCATGTTGGATTTTGAGTGCTGCTGTG--TT    494
Ppa  TTTCTTTTTT---TTAAAGGGAAA---TTGTTGGATTTTGAGTGTTGCTGTTGCTT    475
                                     <----  Ppa2'er
Pme  TTAATATAGCTCACTTTAAATAAATAAATATATAAA---TTCTTGTATATATATAT    547
Ppa  TTTTTGCAGCTCACTTTAAATAAATAAATATATATAAGTTTC-AGTATAT---TTT    527

Pme  GGTGTAATAATAACAAACATTTCATCATTAATTTATATAAAGGAAT-ATATATAGT    602
Ppa  GATGTAATAATAA-AATCATTTCATCAAAAAAATAAATATATG--TGAGATTTATT    580

Pme  ATTAAATTATTATT--AT-TAAATTTT----AAGACCTCAAATCAGGTG-GACTAC    650
Ppa  ATAACATTA--ATTGAATGTAAATTTTTTTTAAGACCTCAAATCAGGTGAGACTAC    634

Pme  CCACTGAACTTAAGCATATCAATAAGCGGAGGA                           683
Ppa  CCACTGAACTTAAGCATATCAATAAGCGGAGGA                           667
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Figure 3: Real-time PCR strategy for the amplification of diagnostic PCR products using pathogen-specific oligonucleotide primers from the ITS regions. The primer pair Ppm1 (orange box)/Ppa2 (pink box) yields a PCR product of 141 bp that is specific for *P. pachyrhizi*. The combination of PCR primers Ppm1 (orange box)/Pme2 (blue box) generates a PCR product of 138 bp that is specific for *P. meibomiae*. Both assays use the same FAM-labeled probe (purple box).

Figure 4: Real-time PCR strategy for the amplification of diagnostic PCR products using pathogen-specific oligonucleotide primers from the mtDNA region. The primer pair Tin3 (yellow box)/Tin10 (green box) yields a PCR product of 212 bp that is specific for *T. indica*. The combination of PCR primers Tin11 (blue box)/Tin10 (green box) generates a PCR product of 212 bp that is specific for *T. walkeri*. Both assays use the same FAM-labeled probe (pink box).

Real-time PCR Assays:

Oligonucleotide primers specific to *T. indica*, *T. walkeri*, *P. pachyrhizi* or *P. meibomiae* were synthesized (Life Technologies/Gibco BRL) as described (1,2). The internal oligonucleotide probes (25-mers) were labeled at the 5´-end with the fluorescent reporter dye, 6-carboxy-fluorescein (FAM) and at the 3´-end with the quencher dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). The real-time PCR reactions were conducted using 25 µL total volume and analyzed for 45 cycles using a Smart Cycler TD (Cepheid, Sunnyvale, CA) following methods recommended by the manufacturer. Optimal assay conditions were: 95 °C for 5 s, 60 °C for 60 s, and 72 °C for 5 s for the *T. indica*-specific assay and 95 °C for 15 s and 60 °C for 30 s for the *P. pachyrhizi*-specific assay.

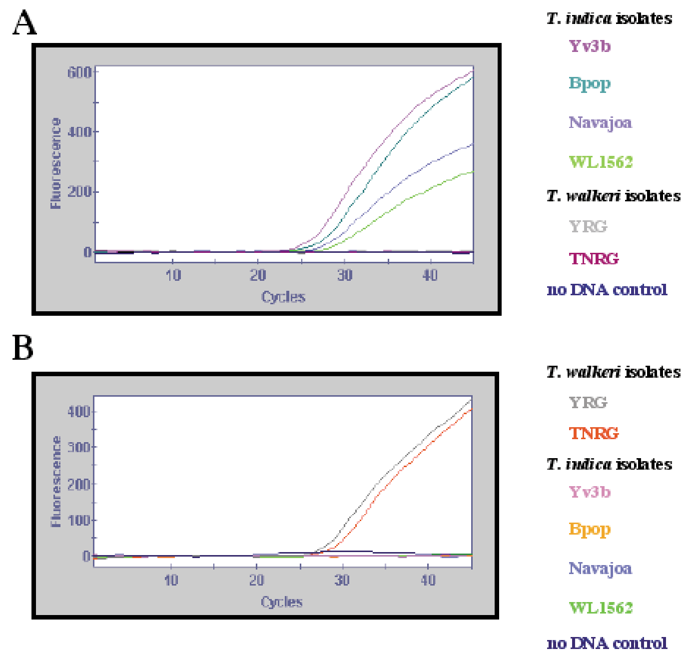

Figure 5: Kinetics of real-time PCR reactions using the Smart Cycler system. *T. indica*-specific primers Tin3/Tin10 were used in the upper panel (A), while *T. walkeri*-specific primers Tin11/Tin10 were used in the lower panel (B). Both assays were conducted in combination with a homologous internal oligonucleotide probe labeled with the fluorescent dye FAM, and fluorescence was monitored after each cycle of PCR. Representative isolates of *T. indica* and *T. walkeri* are shown.

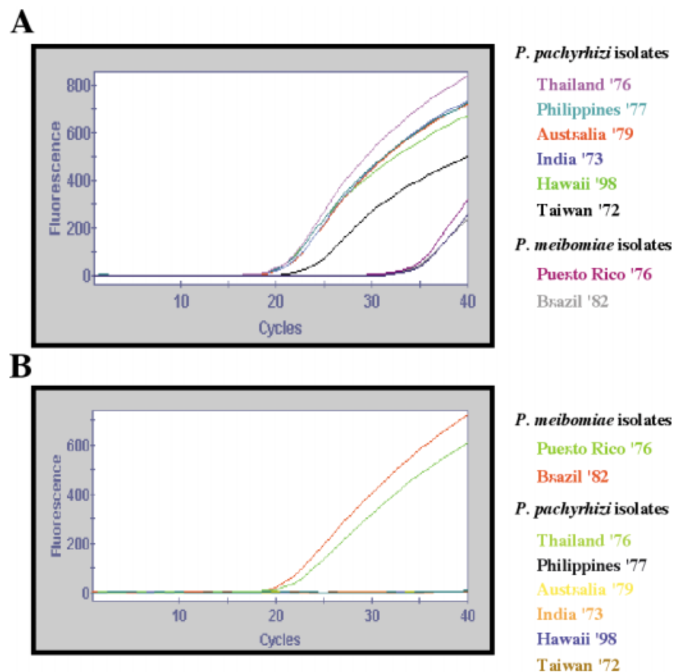

Figure 6: Kinetics of real-time PCR reactions using the Smart Cycler system. *P. pachyrhizi*-specific primers Ppm1/Ppa2 were used in the upper panel (A), while *P. meibomiae*-specific primers Ppm1/Pme2 were used in the lower panel (B). Both assays were conducted in combination with a homologous internal oligonucleotide probe labeled with the fluorescent dye FAM, and fluorescence was monitored after each cycle of PCR. Representative isolates of *P. pachyrhizi* and *P. meibomiae* are shown.

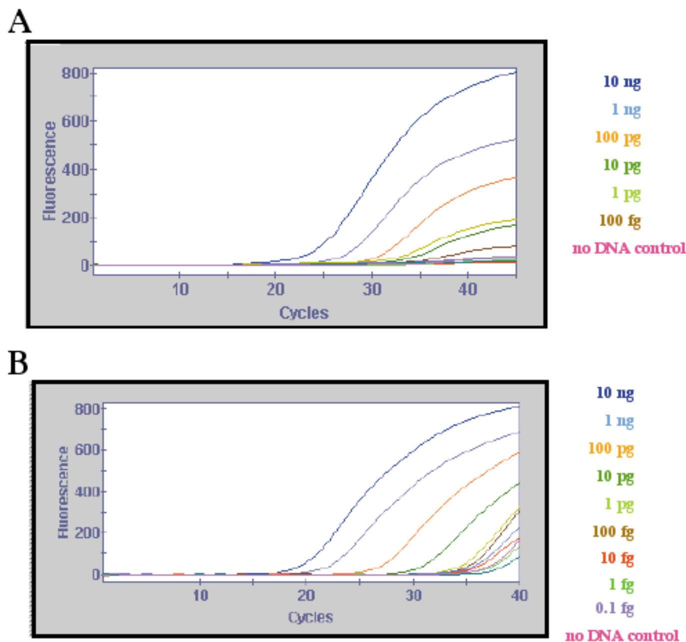

Figure 7: Determination of the amount of template DNA required for real-time PCR using the Smart Cycler for the *T. indica*-specific (A) and the *P. pachyrhizi*-specific (B) assays. Dilutions of genomic DNA of *T. indica* isolates WL1562 and *P. pachyrhizi* isolate Taiwan '72 were used.

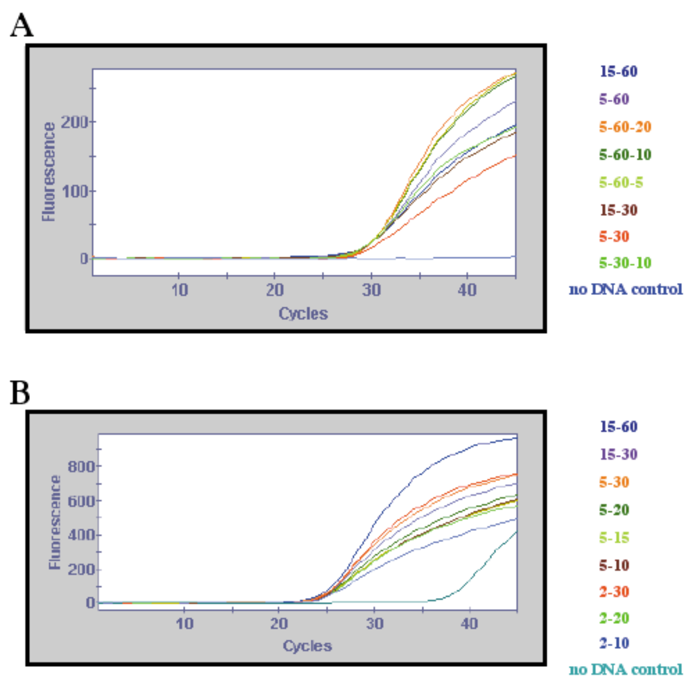

Figure 8: Optimization of the real-time PCR cycling conditions using the Smart Cycler for the *T. indica*-specific (A) and the *P. pachyrhizi*-specific (B) assays. Plots show the duration of the denaturation and annealing cycles (2-step), and elongation cycle (3-step).

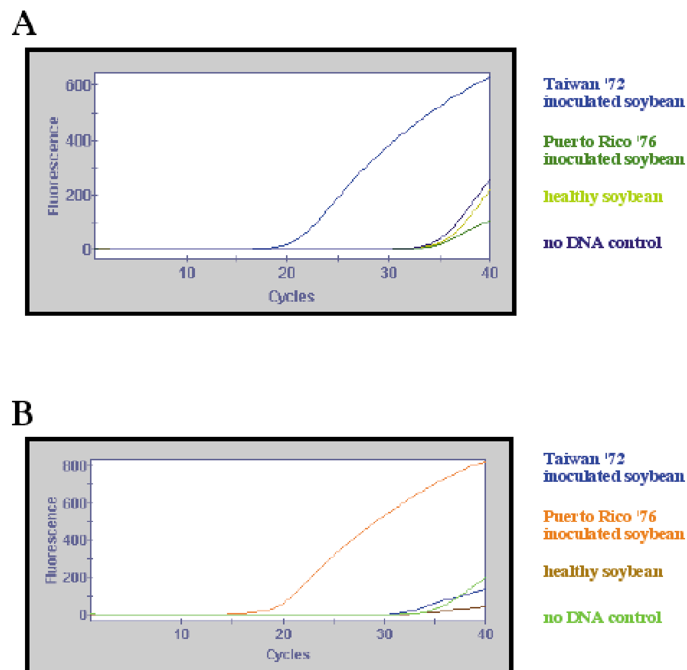

Figure 9: *In planta* real-time PCR assays. Soybean plants were infected with *P. pachyrhizi* isolate Taiwan '72 or *P. meibomiae* isolate Puerto Rico '76. Ten nanograms of genomic DNA from infected plants was used in each assay with *P. pachyrhizi*-specific primers Ppm1/Ppa2 (A) and *P. meibomiae*-specific primers Ppm1/Pme2 Ppa2 (B).

Summary

- The real-time PCR assays for *T. indica* (Karnal bunt) and *T. walkeri* (ryegrass smut) were optimized for use on the Smart Cycler using DNA extracted from mycelia from germinating teliospores.
- The real-time PCR assays for the soybean rust pathogens *P. pachyrhizi* and *P. meibomiae* were optimized for use on the Smart Cycler using DNA extracted from ungerminated urediniospores and infected soybean leaf tissue.
- Real-time PCR assays were completed in approximately 1 hour using the Smart Cycler.

References

1. Frederick, R.D. Snyder, K.E., Tooley, P.E., Berthier-Schaad, Y., Peterson, G.B., Bonde, M.R., Schaad, N.W., and Knorr, D.A. 2000. Identification and differentiation of *Tilletia* indica and *T. walkeri* using the polymerase chain reaction. *Phytopathology* **90:** 951–960.
2. Frederick, R.D., Snyder, C.L., Peterson, G.B., and Bonde, M.R. 2000. Detection and discrimination of the soybean rust pathogens *Phakopsora pachyrhizi* and *P. meibomiae* using PCR. *Phytopathology* **90:** S25.

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