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SESSION 4: RESEARCH ADVANCES IN COPING WITH EMERGING INVASIVE SPECIES IN THE GREATER CARIBBEAN

IMPROVED DISEASE DIAGNOSIS: DEVELOPMENT OF A NOVEL MOLECULAR TECHNIQUE FOR THE DETECTION AND IDENTIFICATION OF PLANT PATHOGENS IN HOST TISSUE

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ABSTRACT

The detection and identification of economically important plant pathogens is of paramount importance in Florida and the Caribbean region. Invasive pathogens are particularly grave threats, as exemplified by the recent establishment of citrus canker, soybean rust, citrus greening, texas phoenix palm decline, and laurel wilt diseases in Florida. Accurate and rapid identification of such threats is fundamental to protecting agriculture in Florida, the United States, and Puerto Rico. This research is designed to increase the diagnostic capacity to reduce and possibly eliminate the harmful effects of such invasive species as listed above that have invaded the region or are a future threat to the region, and adversely affect production agriculture and the natural environment. Plant diagnostics play a crucial role in agriculture and ultimately impact society as a whole. Plant diagnosticians face numerous challenging situations, most of which have a major economic impact, such as diagnosing a non-endemic potentially invasive pathogen at a port of entry. In some instances, there may be life-threatening situations like identifying a poisonous plant or mushroom that was eaten by human or animal. Whatever the case may be, possessing the capability to provide clientele with accurate and reliable diagnoses should be a high priority for all diagnostic laboratories. Therefore, we present a novel PCR technique that, when used correctly, can detect and amplify plant pathogenic DNA directly from host tissue that is several orders of magnitude more sensitive than the Standard PCR. The potential use of the High-Fidelity PCR in routine plant diagnostics is readily obtainable, requiring the same equipment and expense used for the Standard PCR. This new technology has the potential to be developed into a diagnostic tool with unprecedented sensitivity and power to protect agriculture.

INTRODUCTION

Eighty percent of the cut flowers and propagative plant materials that enter the United States arrive in Florida as air cargo, and the volume of perishable products imported into the state has doubled every six years. In 2007, the plant inspection station at the Port of Miami (Florida) processed 74% of all plants imported into the United States, making it the busiest port in the nation. These plants represent many different parts of the world, including the tropics (AQAS Database, USDA/APHIS PPQ 2008). Thus, it is not surprising that the "Florida Pathway" is by

far the most important route of entry for high-risk pathogens and pests into the United States; no fewer than 4–5 introductions of significant plant pathogens occur each year in Florida. Puerto Rico is an important port of entry for agricultural commodities in the Caribbean, as well as a major exporter of propagative plant materials and agricultural products from the United States. Although figures are not available, the Commonwealth is also an important point of entry for new pathogens and pests.

The introduction of significant numbers of plant pathogens into Florida and Puerto Rico go unrecognized due to limitations in visual and current molecular protocols to identify regulated species. The plant diagnostic clinic at the University of Puerto Rico receives on average 100 samples from various crops every month, only 5% of those samples are sequenced for species identification. Another 10% of the samples are diagnosed as unknown etiology and remain unidentified. While numerous plant diagnostic samples processed by clinics within the Florida Plant Diagnostic Network are diagnosed as "no pathogens found" (Palmateer, personal communication), some of these samples may in fact be infected by a pathogen, thus emphasizing the need for more sensitive and effective measures for plant diagnostics.

Although a plant may exhibit symptoms that indicate the presence of a bacterial, fungal, or viral pathogen, in many cases no such symptoms exist, even though the pathogen may still be present in a latent state and go undetected by plant inspectors and diagnosticians. Environmental factors such as temperature and moisture are extremely critical in the disease process and more often than not pathogens remain dormant in plant tissue (Swanson et al. 2007). During this phase of dormancy, it can be very difficult to isolate the pathogen using conventional diagnostic methods (i.e., tissue plating on artificial media), which may lead to a misidentification due to the presence of secondary fungi and bacteria or false negatives, either of which could be catastrophic in situations involving select agents. In addition, many pathogens are obligate parasites and cannot be cultured on artificial media, thus requiring molecular diagnostic techniques like the Polymerase Chain Reaction (PCR) for confirmation. However, plant cellular contents (organic and inorganic compounds), including host genomic DNA, can interfere with the efficiency of Standard PCR (Vickers and Graham 1996; Vincelli and Tisserat 2008; Wilson 1997), making the diagnosis of plant disease directly from plant tissue using the PCR extremely difficult and one of the most common limiting factors for obtaining accurate results.

The polymerase chain reaction (PCR) has become widely used since the discovery (Chien, Edgar, and Trela 1976) and subsequent use of heat-stable DNA polymerase for in vitro replication of DNA (Saiki et al. 1988). This alleviated the tedious task of adding fresh DNA polymerase at the beginning of each PCR cycle (Mullis and Faloona 1987), which led to the automation of the procedure through the production of programmable thermocyclers. The PCR is now used routinely to amplify DNA for phylogenetic studies (Chaverri, Samuels, and Hodge 2005; Dettman, Jacobson, and Taylor 2006; Stewart et al. 1999; Crous, Kang, and Braun 2001; Palmateer, McLean, and Morgan-Jones 2003), genomic analysis (Arneson, Hughes, Houlston, and Done. 2008; Nadeau et al. 1992; Lashkari, McCusker, and Davis 1997), and plant disease diagnosis (Yokomi et al. 2008; Trout et al. 1997), and is used to examine genetic diversity within populations of plant pathogens (Zhang, Fernando, and Remphrey 2005; Urena-Padilla et al. 2002; Winton, Hansen, and Stone 2006). However, the Standard PCR is not efficient at producing long sequences, and is generally unable to produce sequences of more than 5 kb

(Barnes 1994). The High-Fidelity PCR (=Long PCR), which incorporates a second heat-stable DNA polymerase with 3'-exonuclease activity, has been shown to produce longer sequences than Standard PCR, with a product size of up to 35-kb (Barnes 1994). The addition of the proofreading enzyme to the reaction containing an n-terminal deletion mutant of *Taq* polymerase was shown by Barnes (1994) to remove mismatched base pairs, allowing strand synthesis to proceed. The use of the proofreading enzyme alone did not amplify the target DNA, which may have occurred because of the degradation of the primers by the 3'-exonuclease activity of the enzyme when used in excessive amounts (Barnes 1994).

In addition to producing longer sequences than the Standard PCR, the High-Fidelity PCR has been shown to efficiently amplify target DNA while in the presence of large amounts of genomic DNA, which can be from a host organism or from the target organism. Vickers and Graham (1996) were able to use a High-Fidelity PCR protocol utilizing a DNA polymerase mixture containing *Taq* polymerase and a heat-stable DNA polymerase with proofreading ability *(Pwo)* to amplify a single copy gene (*Bar*), a marker for the selection of transgenic plants, while in the presence of Barley genomic DNA. The High-Fidelity PCR consistently amplified the target gene, while the Standard PCR only occasionally produced results. The High-Fidelity PCR has also been shown to detect bacterial infections and microbial associations in numerous arthropod species. Jeyaprakash and Hoy (2000) demonstrated that the High-Fidelity PCR was more sensitive than the Standard PCR in detecting *Wolbachia* infections in arthropods. When plasmids containing the *wsp* gene were amplified while in the presence of arthropod DNA, the High-Fidelity PCR consistently amplified 1 fg of plasmid DNA containing the *wsp* gene while the Standard PCR could detect 1 ng of plamid.

Hoy, Jeyaprakash, and Nguyen. (2001) also showed that High-Fidelity PCR was more sensitive than Standard PCR in detecting the citrus greening pathogen '*Candidatus* Liberibacter asiaticus' while in the presence of genomic DNA from citrus psyllids, citrus trees, or citrus psyllid parasitoids. Furthermore, High-Fidelity PCR has been used to detect and characterize a new Microsporidium species from the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Becnel et al. 2002), to identify and distinguish two parasitoids of the brown citrus aphid (Persad, Jeyaprakash, and Hoy 2004), to examine the microbial diversity of *Metaseiulus occidentalis* and its prey, *Tetranychus urticae* (Hoy and Jeyaprakash 2005), and to amplify 16S ribosomal sequences of endotoxin producing bacteria in varying amounts of dust mite DNA (Valerio et al. 2005).

OBJECTIVES

Juana Diaz receives diseased plant samples representing a multitude of pathogens from the Florida Extension Plant Diagnostic Clinic at the Tropical Research and Education Center and from the diagnostic clinic at the University of Puerto Rico. These clinics are a valuable resource for the agricultural community by providing pathogen identification and disease management recommendations where appropriate, and are a viable means for researchers to monitor plant pathogen populations in the surrounding areas. We used this opportunity to investigate the following research objectives, and thus the following procedures relied on both clinics for plant samples analyzed to:

1. Compare the sensitivity of the High-Fidelity and the Standard PCR procedures by inoculating a range of plants with several fungal and bacterial pathogens and amplifying the target organism's DNA directly from plant tissue.
2. Determine whether the High-Fidelity PCR is more sensitive than the Standard PCR in detecting fungi, bacteria, and viruses from naturally infected plant species.

MATERIALS AND METHODS

DNA was extracted using DNA extraction protocol 3 (below) and amplified using the Standard PCR and High-Fidelity PCR protocols (explained below), and the results compared. To quantify the differences between Standard and High-Fidelity PCR, the serially diluted plasmid DNA (1000 ng to 1 fg) was spiked with 10ng of plant genomic DNA. Two negative controls were used: one containing the plant genomic DNA alone and the other without DNA. All reactions wwere replicated three times.

DNA Extraction Protocols

Two protocols were used to extract genomic DNA from plant host tissue.

Protocol 1: Plant DNA: 3.866 grams of leaf tissue were frozen in liquid nitrogen and ground in 8 ml of CTAB buffer (2% cetyltrimethylammonium bromide, 100 mMTris pH 7.5, 20 mM EDTA, 1% polyvinyl pyrrolidone, and 1.4 M NaCl) for 10 minutes before being aliquoted into 8 tubes. The samples were then incubated at 60°C for 16 hours. After two chloroform extractions, the 8 DNA aliquots were combined into 4 aliquots and precipitated in 2-propanol, and then re-suspended in 100 µL sterile water. All samples were pooled to make a 400 µL sample of genomic orchid DNA. Plant DNA was quantified using an Eppendorf BioPhotometer G131 V1.35 (Eppendorf, Hamburg, Germany).

Protocol 2: The leaf spot fungus *Pseudocercospora odontoglossii* was isolated from a *Cattleya* hybrid and identified based on morphological characters (Ellis 1976; Crous and Braun 2003) and host. Single spores were grown on V-8 juice agar for 2 weeks at 25˚C under artificial light at 12L:12D photoperiod. A section of mycelium approximately 2x2 cm was scraped from the surface of the plate with a sterile wooden applicator stick and placed in a 0.5 mL tube. Then the following procedures were performed: 100 µL of Extraction Solution (Extract-n-Amp, Sigma-Aldrich, St. Louis, MO) was added and the sample was ground for 5 minutes with a sterile plastic pestle and heated to 95˚C for 10 minutes; 100 µL of dilution solution (Extract-n-Amp, Sigma) was added to the sample, after which the sample was briefly vortexed; and 30 µL of the extracted DNA was added to 270 µL of sterile water (Harmon, Dunkle, and Latin 2003).

In order to compare the use of the High-Fidelity PCR and the Standard PCR in the diagnosis of plant diseases, several types of commonly cultivated tropical foliage plants were inoculated with bacterial and fungal pathogens using Standard procedures. DNA from these plants was extracted using a third protocol:

Protocol 3: A cork borer (~6 mm in diameter) was used to cut a section of plant material. The plant section was placed in a 0.5 mL tube and the tissue was frozen in liquid nitrogen and ground

with a sterile plastic pestle for 5 minutes. Then the following procedures were performed: 100µL of extraction solution (Extract-n-Amp, Sigma-Aldrich) was added and heated to 95ºC for 10 minutes; 100µL of dilution solution (Extract-n-amp, Sigma-Aldrich) were added and the sample was briefly vortexed; and 30µL of the extracted DNA was added to 270 µL of sterile water (Harmon, Dunkle, and Latin 2003). After the DNA extraction, the Standard and High-Fidelity PCR reactions were performed using the following protocols with the appropriate primers.

Total Plant RNA Extraction

Total plant RNA was extracted using a modified RiboPure Kit protocol.

- ***Homogenization:***
  1. Homogenize 0.1 g of tissue samples in Liquid nitrogen using sterile pestle and mortar
  2. Re-suspend in 500 µl of TRI Reagent
  3. Incubate the homogenate for 10 minutes at room temperature
  4. Centrifuge at 13,000 rpm for 15 minutes at 4°C; transfer supernatant to a new tube
- ***RNA Extraction:***
  1. Add 100 µL of BCP to 1 mL of homogenate and mix well
  2. Incubate the homogenate for 10 minutes at room temperature
  3. Centrifuge at 13,000 rpm for 15 minutes at 4°C
  4. Transfer aqueous phase to a new 1.5 mL micro centrifuge tube
- **Final RNA Purification:**
  1. Add 200 µL of 100% ethanol and mix immediately
  2. Pass the sample through a filter cartridge
  3. Wash filter twice with 500 µL of Wash Solution; centrifugate 1 minute at 8,000 rpm
  4. Transfer filter to a new eppendorf tube
  5. Elute RNA with 50 µL Elution Buffer and freeze until concentration reading.

Standard PCR protocol

Standard PCR was be performed using DNA extracted from inoculated plants and plants with suspected diseases in a 25µL reaction volume containing 2.5 µL of 10X PCR Buffer +Mg (Boehringer, Mannheim, Germany), 200 µM dATP, dGTP, dCTP, and dTTP, 400pM of primers ITS4 and ITS5 (White et al. 1990) for fungi, and 16S primers (Weisburg et al. 1991) for bacteria and .2 units of *Taq* DNA polymerase (Bioline, Taunton, MA). Samples were covered with 50µL of sterile mineral oil and amplified using the following temperature profile: (i) 94˚C for 5 minutes and (ii) 35 cycles consisting of denaturing at 94˚C for 30 seconds, annealing at 53˚C for 30 seconds, and extension at 72˚C for 1 minute (the described annealing temperature is for the fungal primers).

High-Fidelity PCR Protocol

High-Fidelity PCR will be performed using DNA extracted from inoculated plants and plants with suspected diseases in a 50 µL reaction volume containing 50 mM TRIS, pH 9.2, 16 mM ammonium sulfate, 1.75 mM$MgCl_2$, 350 µL dATP, dGTP, dCTP, and dTTP, 800 pmol of primers ITS4 and ITS5 (White et al. 1990) for fungi and 16S primers (Weisburg et al. 1991) for

bacteria, 1 unit of Accuzume (Roche Molecular Biochemicals) and 5 units *Taq* DNA polymerase (Bioline) (Barnes 1994). Samples were covered with 100 µL of sterile mineral oil and amplified using 3 linked temperature profiles: (i) 94˚C for 2 minutes; (ii) 10 cycles consisting of denaturing at 94˚C for 10 seconds, annealing at 53˚C for 30 seconds, and extension at 68˚C for 1 minute; and (iii) 25 cycles consisting of 94˚C for 10 seconds, annealing at 53˚C for 30 seconds, and extension at 68˚C for 1 minute plus an additional 20 seconds during each consecutive cycle (Hoy, Jeyaprakash, and Nguyen 2001; Jeyaprakash and Hoy 2000) (the described annealing temperature is for the fungal primers).

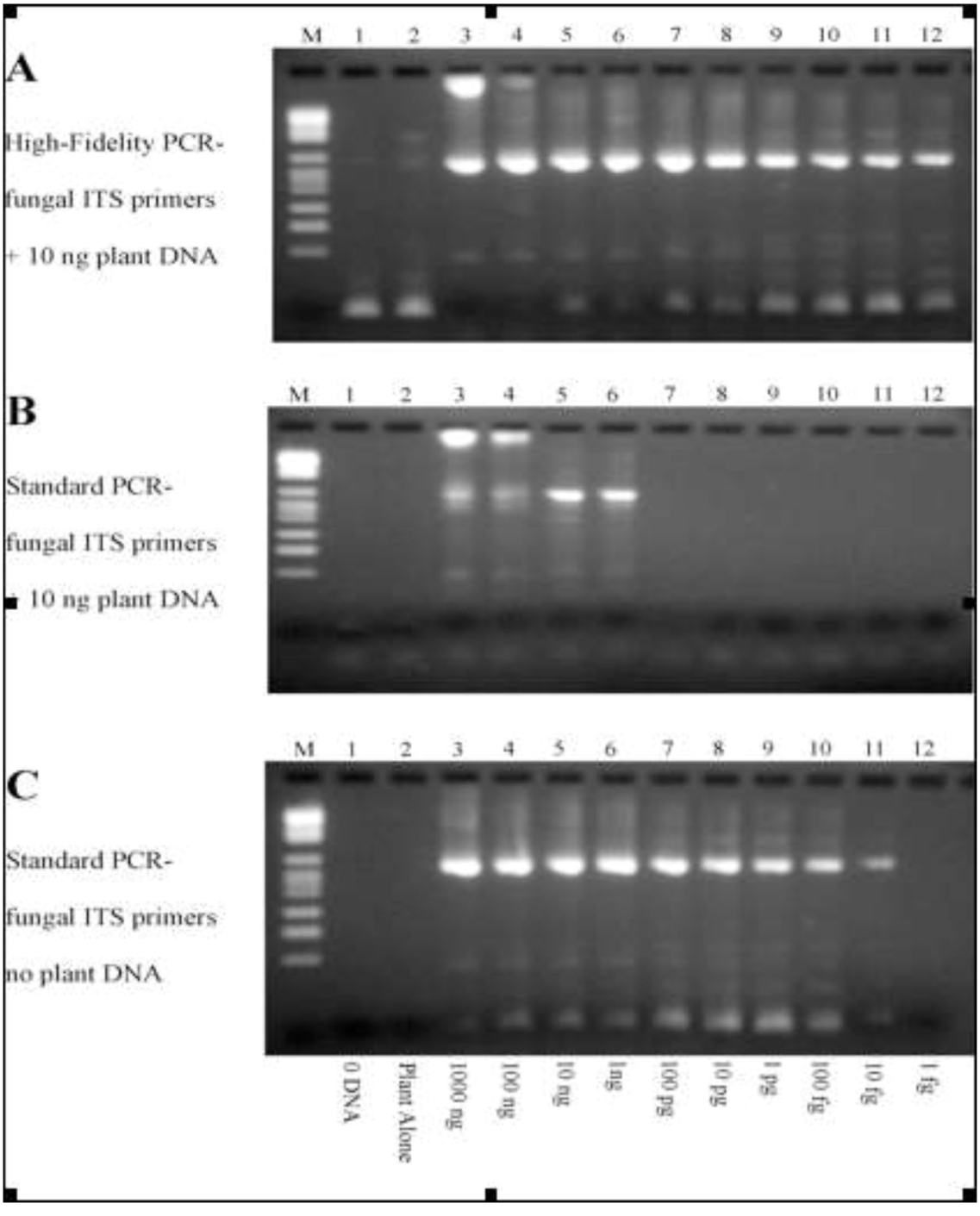
Molecular Cloning for DNA Sequencing

In order to confirm the identity of the obtained PCR product, the target DNA sequences were cloned using the TOPO T/A cloning kit (invitrogen, Carlsbad, CA) and sent for sequencing to the ICBR at the University of Florida (Gainesville). Before ligation into the cloning vector, PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's recommendations and eluted in 50 µL of sterile glass distilled, glass collected water. A 3' A-overhang was added to the PCR product after purification to facilitate ligation into the cloning vector by mixing the 50µL DNA sample with 5.75 µL 10X High-Fidelity buffer(50 mM TRIS, pH 9.2, 16 mM ammonium sulfate, 1.75 mM $MgCl_2$), 100 mMdATP, and 1 unit *Taq* polymerase (Bioline). The reaction was placed in a thermocycler at 72˚C for 45 minutes. The product was immediately cloned into the TOPO T/A cloning vector following the manufacturer's recommendations (Invitrogen Corporation, Carlsbad, CA), and *Escherichia coli* cells were transformed with the recombinant plasmid. *E. coli* colonies were selected from plates containing X-GAL, IPTG, and ampicillin, and grown overnight in LB broth containing ampicillin at 37˚C. Plasmids were extracted using the Qiagen Plasmid Mini Prep Kit (Qiagen, Valencia, CA) and digested with *Eco*RI restriction enzyme, followed by gel electrophoresis on a 2% agarose TAE gel stained with ethidium bromide to confirm the correct size of the insert.

RESULTS AND DISCUSSION

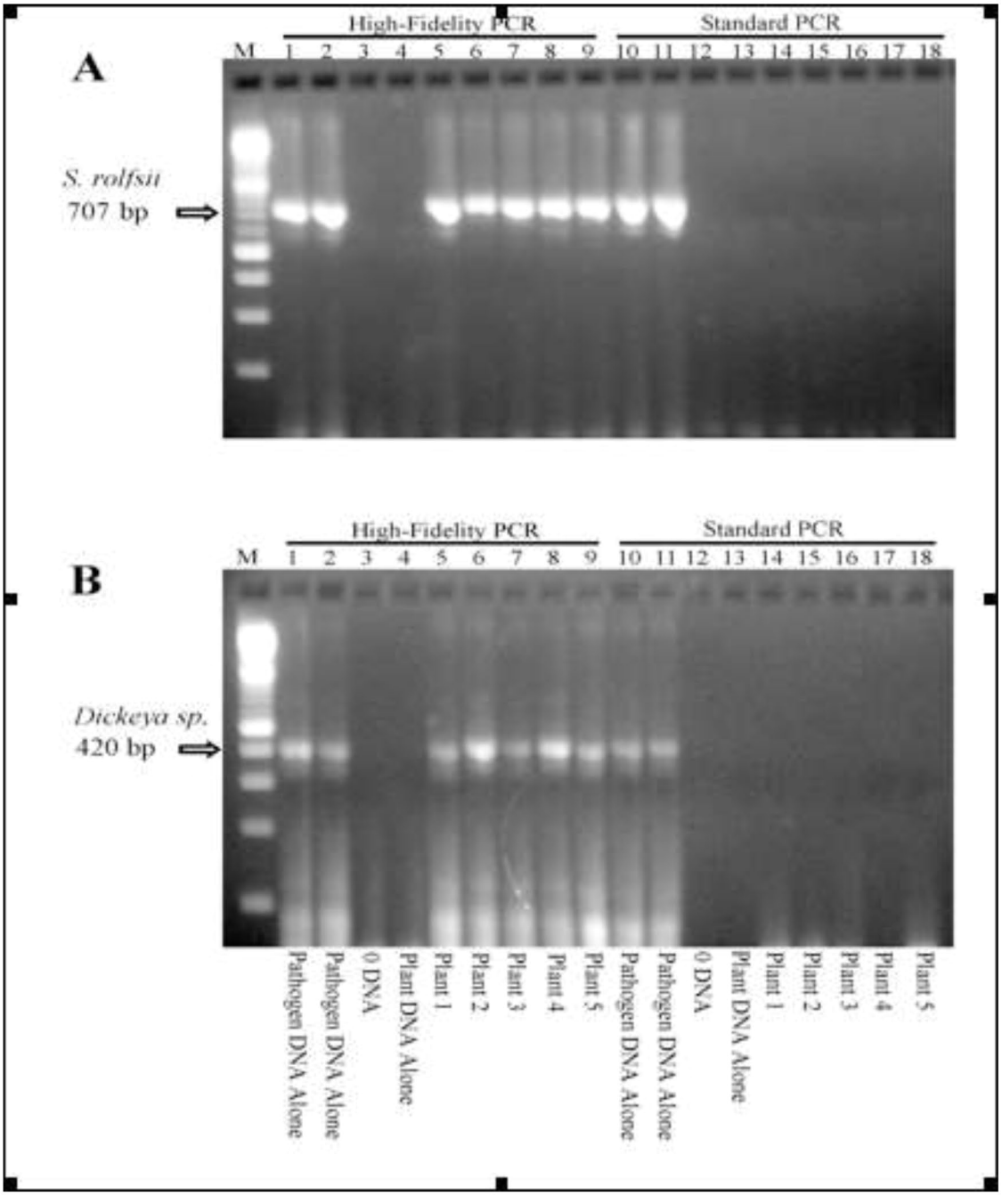
The results of the experiments are shown in Figures 1 through 4.

Figure 1. Comparing High-Fidelity and Standard PCR using plasmid pRC17



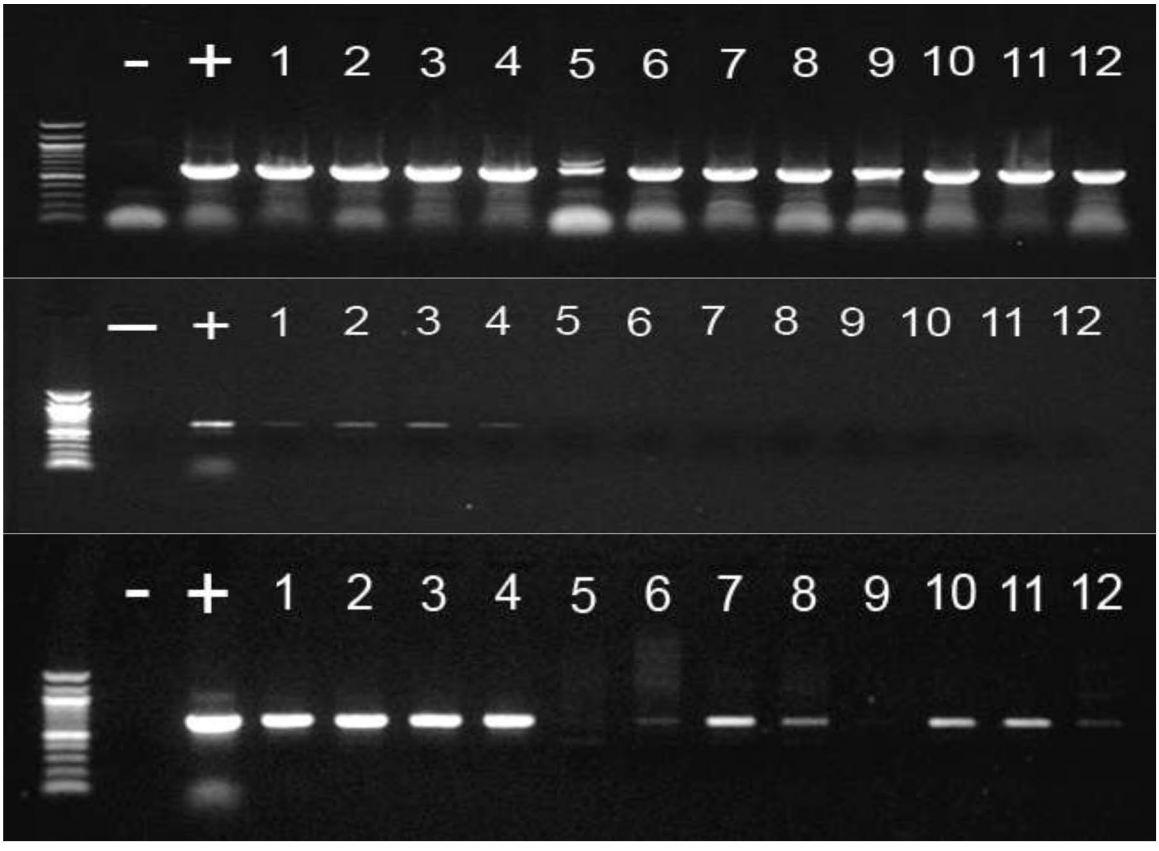
A comparison of High-Fidelity and Standard PCR using plasmid pRC17 containing the 571-bp ITS1, 5.8S, and ITS2 rDNA of *Pseudocercospora odontoglossi* as a template and primers ITS4/ITS5. (**A**) High-Fidelity PCR amplified as few as 207 copies (1 fg) of template in the presence of 10 ng of *Cattleya* DNA, while Standard PCR (**B**) required at least 200 million copies (1 ng) of template for amplification. In the absence of host genomic DNA, the Standard PCR protocol (**C**) amplified as little as 10 fg of template. M=molecular marker IV (Roche).

Figure 2. Comparing High-Fidelity and Standard PCR for two orchid pathogens



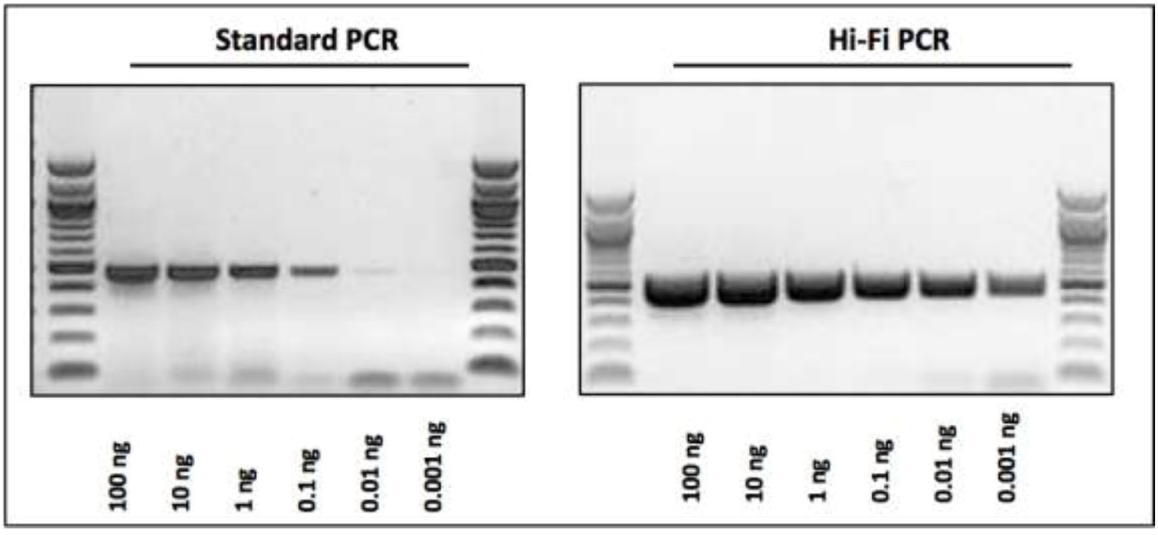
A comparison of High-Fidelity and Standard PCR in the detection of two important orchid pathogens from five inoculated *Phalaenopsis* plants. (**A**) *S. rolfsii* was detected in all five plants using fungal ITS4/ITS5 and High-Fidelity PCR protocol (lanes 5–9), but in none of the plants with Standard PCR (lanes 14–18). (**B**) High-Fidelity PCR detected *Dickeya* sp. in inoculated plants using primers ADE1/ADE2 (lanes 5–9), but not in any plants with Standard PCR (lanes 14–18). M=molecular marker XIV (Roche).

Figure 3. Comparing High-Fidelity and Standard PCR for *P. nicotianae* DNA



Only Hi-Fi PCR using Accuzyme coupled with DNAeasy and Extract-N-Amp extraction positively detected *P. nicotianae* DNA in all samples of all tissues. Accuzyme Hi-Fi PCR also produced positives for the majority of samples using CTAB extraction. LongAmp Hi-Fi PCR detected the pathogen for the majority of samples for all DNA extraction methods except the Shorty protocol. Standard PCR only detected the pathogen for leaf samples using Dneasy extraction. Extraction with Shorty buffer did not produce any positive detection of the pathogen from any sample save a single leaf sample using Accuzyme Hi-Fi PCR.

Figure 4. Comparing High-Fidelity and Standard PCR in RNA extraction



ORSV Primers: (expected band of 474 bp) with Tm 48ºC.
CP-Forward 1: 5' ATGTCTTACACTATTACAGACCCG'3
CP-Reverse 1: 5' GGAAGAGGTCCAAGTAAGTCC '3

Hi-Fi RT-PCR detected 0.001 ng/µl of CymMV and ORSV in a total plant RNA extraction compared to 1ng/ µl of CymMV and 0.1ng/ µl of ORSV, respectively. Thus, Roche Titan One tube kit proved to be more sensitive than conventional RT-PCR amplification. The detection of these viruses was performed on total plant RNA, which implies that when using a viral RNA extraction, these results will likely be enhanced in sensitivity.

These results indicate that High-Fidelity PCR is a more sensitive in detecting pathogen DNA from symptomatic host tissue samples. Our study also indicates that a quick DNA extraction method like the Extract-N-Amp kit produces similar results as a longer method like the DNeasy Kit, even though DNA purity may be lower. The Extract-N-Amp protocol also requires minimal tissue processing.

The advantage of High-Fidelity PCR is that it is similarly easy to run as Standard PCR and does not require special equipment, making it less costly than other molecular detection methods that confer increased detection sensitivity, like real-time PCR. When combined with species-specific primers, this detection method enables diagnosis of specific pathogens directly from plant tissue in a matter of hours, which is especially useful for time sensitive disease problems.

In the future we plan to implement the High-Fidelity detection method for other important pathogens in South Florida, including citrus greening, lethal yellowing of palms, and laurel wilt of avocado.

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