Development and Evaluation of AmplifyRP Acceler8 Diagnostic Assay for the Detection of Fusarium oxysporum f. sp. vasinfectum Race 4 in Cotton

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ABSTRACT


A rapid and reliable molecular diagnostic assay, AmplifyRP Acceler8, was developed for the direct detection of Fusarium oxysporum f. sp. vasinfectum (FOV) race 4, a virulent genotype of the Fusarium wilt pathogen of cotton (Gossypium spp.), in soil and cotton tissue. Unlike traditional polymerase chain reaction (PCR) assays, the recombinase polymerase amplification based-assay described here utilizes an advanced isothermal technology where the amplification is carried out at a single constant temperature, 39°C, without the need of a thermal cycler. The AmplifyRP Acceler8 diagnostic assay consistently detected FOV race 4 from all infected tissue samples. The test is rapid, simple, and more sensitive than conventional PCR. The AmplifyRP Acceler8 diagnostic assay detected DNA from FOV race 4 at concentrations of 1 ng/μL and above. In addition, it did not amplify DNA from other known FOV races (races 1, 2, 3, 6, and 8). The whole process from sample preparation to reading the results can be completed in as little as 30 min. The test can detect FOV race 4 from cotton taproots, petioles, and stems.

INTRODUCTION

Fusarium wilt of cotton, caused by the soilborne fungus Fusarium oxysporum Schlechtend.:Fr f. sp. vasinfectum (Atk.) W.C. Snyder & H.N. Hansen, is a widespread disease occurring in most cotton-growing areas of the world. The disease was first described by Atkinson in the United States in 1892 (5). Currently, there are six nominal races: 1, 2, 3, 4, 6, and 8, as well as many unnamed genotypes worldwide (5,9). Many are widespread in the United States, but race 4 (FOV R4), a highly virulent race, is currently the most devastating in many areas of the state; therefore, the potential for spread of FOV race 4 by seed is high (2). Currently there are no effective seed treatments available to eliminate FOV from infested seeds. To aid in the prevention of movement of FOV race 4 in seed and soil, rapid and reliable diagnostic tools are urgently needed to identify affected fields. Conventional polymerase chain reaction (PCR) using race 4-specific primers, R4f (5’GCTCCGTGTCWGAGCCTTCTT) and R4r (5’GTTATGCTCCACGATGAGCA), is presently used to detect the presence of race 4 in cotton by a few laboratories (18). This method of detection can be completed within a day but requires a well-equipped laboratory. To meet the increasing demand for a rapid and simple test for race 4 in cotton tissue and soil, we recently developed the AmplifyRP Acceler8 assay, an isothermal DNA/RNA detection platform that uses recombinase polymerase amplification technology (13). This isothermal amplification has been used for the detection of various plant pathogens such as Fusarium graminearum (12). It requires two target-specific primers, a 5’ end primer and a biotin-labeled 3’ end primer, and one FITC-labeled internal probe. The specific recombination between the target’s DNA and the primers or probe, and subsequent amplification reaction are carried out at a single isothermal temperature (39°C) through multiple enzymes including a recombinase, a polymerase, and an endonuclease (6,19). Because only a single constant temperature is required, the test can be performed in the field within 30 min.

REAGENTS AND SAMPLE PREPARATION

The total reagent mixture for the AmplifyRP Acceler8 assay (proprietary) was lyophilized into powder in a 200-μL PCR tube and called AmplifyRP Acceler8 Reaction Pellet (abbreviated “pellet”) (Agdia Cat# ASP19700). The pellet contained all the reaction components, which included the FOV race 4-specific primers and an internal probe (proprietary). The primers were...
based on the R4f and R4r primers with modification related to the isothermal procedure. The PD1 Pellet Diluent (Agdia Cat # ACC00480) and sample DNA were added immediately before the reaction was started. To prepare sample DNA, 300 mg of fresh cotton stem tissue with vascular discoloration was ground in a sample mesh bag containing 3 ml of GEB3 buffer (Agdia cat# ACC00360). Ten microliters of PD1 buffer was transferred into a 0.2 ml microcentrifuge tube containing the pellet. One microliter of the sample DNA was immediately added and mixed with a vortex mixer. The amplification reaction was incubated at 39°C for 15 min. Next, the unopened tube was placed into the Amplicon Detection Chamber (Agdia Cat# ADC98800) (Fig. 1). The whole detection apparatus was then assembled and the result was observed within 20 min.

CONFIRMING SPECIFICITY TO RACE 4

To confirm the specificity of the assay, cultures of FOV isolates FOV-5 race 1, FOV-16611 race 2, FOV-11 race 3, FOV-1201 race 4, FOV-36198 race 6, and FOV-7 race 8 were revived from colonized pieces of Whatman filter paper (no. 1001125, Maidstone, United Kingdom), and cultured on acidified potato dextrose agar (APDA) for 7 days at 24°C. Two hundred mg of fungal mycelia, scraped from an APDA plate, was ground in a sample mesh bag containing 3 ml of GEB3 buffer. This homogenate was then directly applied to the AmplifyRP Acceler8 kit (Agdia Cat# 19700). Each assay was replicated four times and each assay set was conducted three separate times for a total of 12 assays per culture. In addition, conidial suspensions were prepared from 1-wk-old APDA cultures of FOV races 1, 2, 3, 4, 6, or race 8 on 9 cm-diameter petri plates by flooding with 15mL autoclaved deionized water, dislodging the conidia with a glass slide and filtering the suspension through four layers of cheesecloth. The concentration of the conidial suspension was determined with a hemocytometer and adjusted to 1 × 10⁷ conidia per ml with autoclaved deionized water. The conidial suspension was used to inoculate plants.

Cotton plants inoculated with conidial suspensions of the different races of FOV were also assayed. To inoculate plants, roots of 2-wk-old Deltapine 744 (Gossypium barbadense L.) seedlings, a cultivar highly susceptible to race 4 (abbreviated “DP744”), were dipped for four min in a conidial suspension (1 × 10⁷ conidia per ml) from cultures of FOV races 1, 2, 3, 4, 6, or water (non-infected control) and transplanted into plastic pots 10 cm in diameter (750 ml) containing UC potting soil mix (7). Seedlings of Phytojen 800, a cotton cultivar resistant to FOV race 4, was also inoculated with a conidial suspension of FOV race 4. Four weeks postinoculation, 300 mg of fresh stem tissue near the soil line from plants with symptoms typical of Fusarium wilt (wilting, dark brown vascular discoloration, and interveinal and marginal leaf chlorosis and necrosis) was ground in a sample mesh bag containing 3 ml of GEB3 buffer. This crude homogenate was then directly applied to the AmplifyRP Acceler8 kit. Four plants infected with each race or the water control were assayed and the test was conducted three separate times on different plants.

The AmplifyRP Acceler8 assay detected FOV race 4 (a testing line on the test strip indicated a positive reaction) in all FOV race 4 mycelial preparations, conidial preparations, and stem tissues (Fig. 2).

FIELD SAMPLES

Pima cotton plants (Phytogen 805RF) with symptoms typical of Fusarium wilt were collected from four FOV race 4-infested commercial fields in Kern County, California. FOV race 4 had been previously confirmed in the four commercial fields by PCR and pathogenicity tests (data not shown). In addition, healthy Pima cotton plants that did not display symptoms typical of Fusarium wilt were collected from two different fields free of FOV race 4 in the same area. Three hundred mg (fresh weight) of stem tissue with brown vascular discoloration was ground in a sample mesh bag containing 3 ml of GEB3 buffer. This crude homogenate was then directly applied to the AmplifyRP Acceler8 kit. The same plants tested with the AmplifyRP Acceler8 kit were evaluated with conventional PCR using race 4-specific primers (R4f and R4r) by direct DNA extraction from vascular tissue and DNA extraction from Fusarium cultures isolated from taproot tissue. To isolate FOV, roots were washed with anti-bacterial soap, surface sterilized in 0.6% sodium hypochlorite (10% bleach) for 1 min, and placed on APDA plates. After 5 days, DNA was extracted from Fusarium-like colonies using Qiagen DNeasy Plant Mini Kit (Valencia, CA) according to the manufacturer’s

**FIGURE 1**
Detection of FOV race 4 in infected cotton with an AmplifyRP Acceler8 amplicon detection chamber.

**FIGURE 2**
Detection of FOV race 4 in inoculated Deltapine 744 cotton tissue by an AmplifyRP Acceler8 Kit. FOV races 1, 2, 3, 6, and 8 resulted in no reaction. Plants mock-inoculated with water served as the healthy control.
Acceler8 conducted three separate times. From each of the six fields were assayed and the assay was conducted three separate times.

FOV race 4 was positively identified by the AmplifyRP Acceler8 kit in each of the 48 assays using symptomatic root tissue from the four infested fields. FOV race 4 was not detected in 24 samples from asymptomatic root tissues from the two fields free of FOV race 4. The same results were observed with direct DNA extraction from vascular tissue and DNA extraction from Fusarium cultures isolated from taproot tissue (data not shown).

DETECTION OF FOV R4 IN VARIOUS PLANT TISSUES

Three hundred mg was collected from each of the following tissues from FOV R4-inoculated and noninoculated greenhouse-grown plants: roots with vascular discoloration; petioles of the first vegetative node (mainstem node 1); leaf blades from the first vegetative node; stem tissue without vascular discoloration between mainstem nodes 1 and 2 or mainstem nodes 2 and 3; stem tissue without vascular discoloration between mainstem nodes 4 and 5; and tissue without vascular discoloration from the terminal node. Samples were ground in a sample mesh bag containing 3 ml of GEB3 buffer. This crude homogenate was then directly applied to the AmplifyRP Acceler8 kit. The same plant tissues were evaluated with conventional PCR using race 4-specific primers by direct DNA extraction and on cultures isolated from plant tissue as previously described. All assays used four replications and each set of tests was conducted three times.

FOV race 4 was positively identified by the AmplifyRP Acceler8 kit in each of the 12 assays using symptomatic root tissue with vascular discoloration from inoculated plants (Fig. 3). FOV race 4 was not detected in leaf blades from the first vegetative branch, but it was detected 3 of 12 times from the petiole of the first vegetative node. FOV race 4 was detected 5 of 12 times from stem tissue without vascular discoloration between mainstem nodes 1 and 2 or mainstem nodes 2 and 3. The fungus was never detected in 12 attempts in stem tissues without vascular discoloration between the mainstem nodes 4 and 5. However, FOV race 4 was detected 1 of 12 times from stem tissues without vascular discoloration of the terminal node. The culture-based isolation method and direct DNA extraction of surface sterile fresh cotton tissue followed by PCR using race 4-specific primers confirmed all of the above results, i.e., the fungus was detected by the AmplifyRP Acceler8 kit only when it was recovered by culture or detected by conventional PCR. In no case was race 4 detected in noninoculated plants.

INOCULATED SOIL SAMPLES

FOV race 4 conidial suspensions were prepared and adjusted to 1 × 10^2, 1 × 10^3, 1 × 10^4, 1 × 10^5, 1 × 10^6, 1 × 10^7 conidia/ml as described previously. Three hundred microliters of each conidial suspension and 300 mg of autoclaved and dried UC potting soil mix were ground in a sample mesh bag containing 3 ml of GEB3 buffer. This crude homogenate was then directly applied to the AmplifyRP Acceler8 kit. Three hundred microliters of each conidial suspension and 300 mg of field soil with no history of cotton culture from the Plant Pathology Department Field Station on the UC Davis campus (Davis, CA) were ground in a sample mesh bag containing 3 ml of GEB3 buffer. This crude homogenate was then directly applied to the AmplifyRP Acceler8 kit. Four replications of each conidial dilution were assayed three separate times.

The AmplifyRP Acceler8 kit detected FOV race 4 in all 12 samples of UCD potting soil amended with 1 × 10^2, 1 × 10^3, 1 × 10^6, or 1 × 10^7 conidia/gram of dried soil. In UCD potting soil inoculated with 1 × 10^3 conidia/gram of dried soil, the fungus was detected in 2 of 12 samples. FOV race 4 was not detected in potting soil inoculated with 1 × 10^2 or 1 × 10^3 conidia/gram of dried soil. In field soil, the AmplifyRP Acceler8 kit detected FOV race 4 in all 12 soil samples amended with 1 × 10^2, 1 × 10^3, 1 × 10^4, and 1 × 10^5 conidia/gram of dried soil. In field soil inoculated with 1 × 10^4 conidia/gram of dried soil, the fungus was detected in 6 of 12 samples. FOV race 4 was not detected in field soil inoculated with 1 × 10^3, 1 × 10^5, or 1 × 10^6 conidia/gram of dried soil.

INFESTED FIELD SOIL

Three hundred mg of soil from commercial fields in Kern County naturally infested with FOV race 4 was ground in a sample mesh bag containing 3 ml of GEB3 buffer. This crude homogenate was then directly applied to the AmplifyRP Acceler8 kit. The same amount of soil was also assayed with conventional PCR using race 4-specific primers by direct DNA extraction and on cultures isolated from field soil with no history of cotton culture from the Plant Pathology Department Field Station on the UC Davis campus (Davis, CA) using the manufacturer’s protocol. There were four soil samples in each assay, which was conducted three separate times.

FOV race 4 was detected by the AmplifyRP Acceler8 kit once in 12 samples of FOV race 4-infested soil. FOV race 4 was detected by the direct DNA extraction two of 12 times, while it was detected 10 of 12 times when the fungus was recovered in culture.
SENSITIVITY OF AGDIA AMPLIFYRP ACCELER8

FOV race 4 cultures were grown on APDA for 7 days at 24°C. DNA was extracted from 200 mg of mycelium scraped from plates with the Qiagen DNeasy Plant Mini Kit using the manufacturer’s protocol. Fungal genomic DNA concentration was measured three times using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and adjusted to 0.5 ng/µL, 1 ng/µL, 2 ng/µL, 5 ng/µL, 10 ng/µL, and 50 ng/µL using sterile deionized water. The average ratio of absorbance at 260 nm and 280 nm was 1.86. One microliter of each DNA concentration was added to 300 mg of noninoculated fresh DP744 root tissue and ground in a sample mesh containing 3 ml of GE3B buffer or sterile deionized water. This crude homogenate was then directly applied to the AmplifyRP Acceler8 kit. Non inoculated DP744 root tissue acted as background DNA. The crude homogenate was also used in conventional PCR as previously described. This crude homogenate was directly assayed using conventional race 4-specific PCR described previously. This was done four times for each conidial suspension dilution and was repeated three separate times.

The AmplifyRP Acceler8 assay detected DNA from FOV race 4 in root tissue at a concentration of 1 ng/µL and above while the conventional PCR assay detected FOV race 4 DNA at 5 ng/µL and above (Fig. 4).

MULTIPLE SAMPLES WITH AGDIA AMPLIFYRP ACCELER8.

A total of 300 mg of symptomatic root tissue of greenhouse-grown, FOV4r4-inoculated DP744 plants was mixed in the following proportions with noninoculated fresh DP744 root tissue and ground in a sample mesh containing 3 ml of GE3B buffer and applied to the AmplifyRP Acceler8 kit. The same assay was conducted with FOV race 4-infected cotton plants (Phytagen 805 RF) from commercial fields. All assays included four replications for each plant sample and each assay was repeated three separate times.

The AmplifyRP Acceler8 kit detected FOV race 4 in all FOV race 4-inoculated DP744 samples, except in the samples that were diluted 1:100. The same results were observed for dilutions of infected field samples.

CONCLUSIONS

The AmplifyRP Acceler8 kit provided a rapid and reliable method to detect FOV race 4 in infected plants. The kit was specific to FOV race 4 and did not detect other tested genotypes of the fungus. The commercial kit (Agdia cat# ACS19700) contains the reaction pellets, PD1 buffer, plant extraction buffer (General Extraction Buffer 3), amplicon detection chambers and instructions. A mesh bag complete with buffer is provided for macerating up to 500 mg of tissue which is then transferred to a 0.5 mL microcentrifuge tube containing the pellet where amplification is carried out at a single constant temperature. The whole process from sample preparation to reading the results can be completed in as little as 30 min. Each assay currently costs about $30 US per sample.

Even though the AmplifyRP Acceler8 kit can detect FOV race 4 from various tissues (roots, stems, and petioles), we recommend assaying symptomatic stem tissue near the soil line. In no case did AmplifyRP Acceler8 result in a false negative or false positive if symptomatic stem tissue near the soil line was used. Results from other tissue, however, can be variable. For example, when using the kit to detect FOV race 4 from petioles, a false negative is possible. The kit is comparable to conventional methods for detecting FOV race 4 in cotton tissue. When detection of FOV race 4 in bulk samples is required, we recommend combining up to 25 stem samples (12 mg each) from the same area of the field. One infected plant combined with 24 healthy plants resulted in a positive reaction in our tests. Positive results were not assured when tissue from one infected plant was diluted with tissue from 49 healthy plants. This procedure can only be recommended if the plant tissue is macerated and mixed uniformly; otherwise, the assay should be limited to a single plant sample.

Although the AmplifyRP Acceler8 kit can detect FOV race 4 in soil, the results were not reliable. Apparently, there may be compounds in the soil that may inhibit the performance of the kit. In addition, we found that a very faint band can be observed with very low DNA concentrations (below 1 ng/µL). In this case, we recommend waiting at least 3 hours for the bands to darken. We have confirmed that a faint band is indicative of a positive result. Based on sensitive assays, the kit is more sensitive at detecting FOV race 4 DNA than conventional PCR. The kit detected DNA from FOV race 4 at concentrations of 1 ng/µL, while conventional PCR was able to detect DNA of FOV race 4 at no less than 5 ng/µL.

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FIGURE 4

Values represent the average number of successful detections of FOV race 4 out of four replications of three independent trials (12 samples). For detection by conventional PCR, we used published FOV race 4 specific primers (R4f: 5’GTTATGCTCCAGATGAG-CA). Error bars are standard deviations.

LITERATURE CITED