Seed Treatments and Detection of *Fusarium oxysporum* f. sp. *vasinfectum* race 4

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DEDICATION

To my Friends, Family and Mentor
For Their Unrelenting Support

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Plant Pathology

Seed Treatments and Detection of *Fusarium oxysporum* f. sp. *vasinfectum* race 4

Abstract

Fusarium wilt of cotton, caused by the soilborne fungus *Fusarium oxysporum* f. sp. *vasinfectum,* is a widespread disease occurring in most cotton-growing regions of the world. Fusarium wilt occurs in all domesticated cotton. Currently, six nominal races are recognized: 1, 2, 3, 4, 6, and 8, as well as many un-named genotypes worldwide. Many are widespread in the U.S., but race 4, which is highly virulent, is apparently restricted to California. Race 4 is found in an increasing number of fields in California due in part to seed-borne dissemination. The first aim of this study was to evaluate the efficacy of hot water treatments alone or in conjunction with fungicides and other treatments to reduce the viability of FOV race 4 in infected cotton seed. The second aim was to develop and evaluate a rapid and reliable molecular diagnostic assay, the AmplifyRP® Acceler8™, for the direct detection of FOV race 4 in cotton tissue. In the seed treatment assay, a 1 hour immersion of seed in water or sterile 30% potato dextrose broth (PDB) at 24°C followed by a 20 minute immersion in a 60°C solution containing four fungicides (azoxystrobin, fludioxonil, thiabendazole, and thiophanate) or thiophanate alone were the most effective pretreatment-treatment combinations in reducing FOV in seed and avoiding loss of seed germination and vigor. The incidence of FOV in the seed was reduced by approximately 86% without reducing seed germination and vigor based on recovery of the fungus on petri plates and greenhouse grow-out assays. FOV was completely eliminated from infected seed when the seed was pretreated in water at 24°C followed by a 20 minute immersion in a solution of thiophanate

heated to 70°C. With this treatment, seed germination was reduced by 36% and vigor was reduced by 38%. 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 was completed in as little as 30 minutes. The test detected FOV race 4 in cotton taproots, petioles, and stems.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Cotton, the most important fiber crop in the world, is one of the oldest and most widely cultivated crops (23, 32, 34). Traces of cotton fiber dated to 5000 B.C. were found in the ancient ruin of Mohenjo-daro in the province of Sindh, Pakistan (11, 23). Cotton is a major crop in many parts of the world; in 2012 it was grown in 80 countries (13, 27). In the Malvaceae family, cotton belongs to the genus *Gossypium*, which consists of approximately 50 species (8, 26, 31). Of these, *Gossypium arboretum* L., *Gossypium barbadense* L. (Pima), *G. herbaceum* L., and *G. hirsutum* L. (Acala and non-Acala Upland) are the most extensively cultivated species (8, 26). Cotton can be grown in a broad range of soil types and environments, ranging from temperate to tropical. Cotton fibers, which arise from the ovule epidermis, are highly elongated single cells largely composed of cellulose. Technically cotton fibers are considered trichomes (25). Cotton is valued both for its fiber and seed. In addition to the value of the lint to the textile industry, seed provides a variety of nutritional products such as cooking oil, cottonseed meal, and hulls that benefit both consumers and livestock (6, 24, 26). For example, cottonseed is a good source of protein; approximately 45% of the seed is used as meal and 27% is used as livestock feed (6). About 16% of the seed is used to make high quality vegetable oil for human consumption. Another 5-8% of the seed is used to sow the next crop (6).

1.2 COTTON PRODUCTION

In 2012, world cotton production was 123.1 million bales (217.7 kg of lint/bale) (27). The People's Republic of China, the Republic of India, and the United States are the top three

cotton-producing countries, accounting for 75% of the world production (27). In 2012, the United States produced 17.3 million bales of cotton valued at approximately $5.9 billion (29). The top three exporters in the world are the United States, India, and Australia, which account for approximately 47% of the world's export market of cotton. In the United States, 13 million bales of cotton were exported in 2012, making the United States the top exporter of cotton in the world (27). The top three importers in the world are the People's Republic of China, Turkey, and Bangladesh, which account for approximate 60% of the world's import market (27). The People's Republic of China imported approximately 20 million bales of cotton, accounting for 44% of world cotton trade in 2012 (13, 27).

According to the National Cotton Council of America, business revenue stimulated by cotton in the U.S. was estimated at $100 billion in 2012 (18). In the United States, cotton is the fifth most widely cultivated crop in terms of planted area after corn, wheat, soybeans and hay (28). In 2013, approximately 4.18 million ha of cropland were devoted to cotton in the U.S (28). In 2013 cotton was grown in 17 states: Texas (2.3 million ha), Georgia (526 thousand ha), North Carolina (170 thousand ha), Alabama (133.5 thousand ha), Arkansas (129.5 thousand ha), Mississippi (129.5 thousand ha), California (113.3 thousand ha, including 75.3 thousand ha of Pima or 96% of the total U.S Pima production (28)), South Carolina (113.3 thousand ha), Missouri (109.3 thousand ha), Tennessee (105.2 thousand ha), Arizona (69.2 thousand ha), Oklahoma (60.7 thousand ha), Louisiana (52.6 thousand ha), Florida (50.6 thousand ha), Virginia (28.3 thousand ha), New Mexico (13.8 thousand ha), and Kansas (12.1 thousand ha).

1.3 FUSARIUM WILT OF COTTON

Fusarium wilt of cotton, caused by the soilborne fungus *Fusarium oxysporum* Schlechtend.:Fr f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans, is a widespread disease

occurring in most cotton-growing regions of the world. Fusarium wilt affects all domesticated cotton varieties (8). The disease was first described by Atkinson in the U.S. in 1892 (8). Currently, there are six nominal races of *F. oxysporum* f. sp. *vasinfectum* (FOV): 1, 2, 3, 4, 6, and 8, as well as many un-named genotypes worldwide (8, 16). Races 1, 2, 3, 6, and 8 are mildly virulent but can cause severe symptoms if plants are also infected with root knot nematodes (*Meloidogyne* spp.) (5, 15, 16, 26). Many races are widespread in the U.S., but race 4, which is highly virulent, is apparently restricted to California (2, 3, 4, 16, 33). Race 4 is of great concern to growers because most cotton cultivars lack resistance. Unlike most other races and genotypes of FOV, race 4 can cause severe symptoms in cotton, especially Pima cultivars (*Gossypium barbadense* L.), in the absence of the root-knot nematode (*Meloidogyne* spp.) (5, 15, 16, 26). Race 4 is found in an increasing number of fields in California due primarily to seed-borne dissemination (2, 3, 17). The disease can quickly become widespread as residue from infected plants release numerous propagules into the soil, potentially increasing disease severity in subsequent cotton crops. In addition, the disease is very difficult to manage. Once a field becomes infested, there are no effective means to eradicate the pathogen from the soil. FOV persists in the soil indefinitely because it can colonize the roots of other crops and weeds (5, 21, 22, 26). In addition, FOV overwinters as chlamydospores, which may survive in the soil for a number of years (21).

1.4 MANAGEMENT OF FUSARIUM WILT OF COTTON

Management of Fusarium wilt of cotton is difficult to achieve and no single control measure is fully effective. Currently, the disease can be managed using crop rotation, cultural practices, and resistant cultivars (8). Crop rotation may not reduce populations of FOV in soil because FOV survives in the rhizosphere of other crops and weeds (5, 8, 22). Control of root

knot nematode with nematicides and root knot nematode-resistant varieties can reduce Fusarium wilt caused by most genotypes with the exception of race 4 and the two virulent genotypes of FOV in Australia (5, 9, 15, 16, 26, 30). Soil treatments such as solarization and fumigation with methyl-bromide + chloropicrin or chloropicrin + 1,3-dichloropropene significantly reduced the number of FOV spores in the soil (5). The use of resistant cultivars to manage Fusarium wilt has been the most successful management strategy. Few commercial cultivars with Fusarium wilt and root knot nematode resistance are available (14, 26). For example, Phytogen 800, Phytogen 802RF, and Deltapine 360 provide a relatively high level of resistance to FOV race 4, while Phytogen 367 WRF, Phytogen 565 WRF, Stoneville 5458 B2RF, and Stoneville 4288 B2RF provide resistance to both FOV race 1 and root knot nematode (19). The cotton industries in Australia, Egypt, Tanzania, and China were successful in managing Fusarium wilt with the use of cotton cultivars resistant to local races of FOV (8, 14).

1.5 SEEDBORNE AND SEED TREATMENTS

The potential for unintended dissemination of infected seed, especially with virulent isolates such as FOV race 4, is a primary concern of the cotton industry. Currently, there are no effective treatments available to eliminate FOV from infested seeds. Thermotherapy alone or in conjunction with chemicals has been used as an effective seed treatment for various pathogens. In one study, *Fusarium moniliforme* was eliminated from infected maize seed when seed was pretreated in distilled water for 4 hours at 22°C and then immersed in a hot water bath for 5 min at 60°C (7). In another study, Fahim et al. found that a hot water treatment for 10-20 min at 60°C followed by a fungicide dressing eliminated *F. moniliforme* in maize seed without affecting seed germination (12). Moreover, the incidence of *F. circinatum* in infected *Pinus radiata* seed was significantly reduced when seed was immersed in a hot water bath for 30 min at 51–52°C (1).

The same result was observed in gladiolus corms infected with *Fusarium oxysporum* f. sp. *gladioli* when cormels were immersed in hot water for 30 min at 53-55 °C (20). du Toit and Hernandez-Perez found that chlorine or hot water seed treatments substantially reduced or eliminated *Cladosporium variabile*, *Stemphylium botryosum*, and *Verticillium dahliae* in spinach seed without damaging seed germination (10). However, published research on the use of thermotherapy as a means to reduce the incidence of FOV in cotton seed is still limited. To date, only one study has evaluated dry heat and hot water treatments as a means to eliminate FOV from cotton seed. Bennett and Colyer reported that seedborne FOV was reduced when seed was incubated in dry heat at 60, 70, and 80°C for 2 to 14 days, or immersed in 90°C water from 45 seconds to 3 min (2). Viability and seed infection of the treated seed was significantly reduced with higher incubation temperatures. Bennett and Colyer found that the incidence of FOV in infected seed was reduced by 80% when seed was immersed for 105 second at 90°C (2). They also found that this reduction was achieved without a significant loss of seed germination and vigor.

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CHAPTER II

Efficacy of Seed Treatments on Viability of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 in Infected Cotton Seed

2.1 ABSTRACT

Fusarium oxysporum f. sp. *vasinfectum* (FOV) race 4 is a seedborne pathogen of cotton. A series of seed treatments using hot water at various temperatures (55 to 90°C) for various lengths of time (105 seconds to 20 min) was tested on seed of both Pima (*Gossypium barbadense*) and Upland (*G. hirsutum*) cultivars to determine the optimum combination to eliminate the pathogen from seed without reducing seed germination and vigor. In 2012, the incidence of FOV race 4 in seed was reduced by ~56% without reducing germination and vigor when FOV race 4-infected cotton seed was immersed in a hot water bath for 20 min at 60°C. In 2013, infected seed was pretreated at 24°C and agitated on a shaker at 100 rpm for 1 hour in 1 L of sterile deionized water or sterile 30% potato dextrose broth (PDB). The seed was then immersed for 20 min at 60°C in 2 L in one of the following: water, 10% solution of household bleach (0.6% NaOCl, w/v), 0.5% sodium lauryl sulfate (SLS), fludioxonil (*Cannonball*® WP), thiabendazole (*Mertect*® 340-F), azoxystrobin (*Quadris*® Flowable), thiophanate (*Topsin*® M 70WP), or a fungicide bath containing all four fungicides. PDB or water pretreatments followed by a 20 minute immersion in a 60°C fungicide slurry containing all four fungicides or thiophanate alone were the most effective pretreatment-treatment combinations in reducing FOV in seed and avoiding loss of seed germination and vigor. The incidence of FOV in the seed was reduced by ~86% without reducing seed germination and vigor based on recovery of the fungus on petri plates and greenhouse grow-out assays. FOV was completely eliminated from infected seed when the seed was pretreated at 24°C followed by a 20 minute immersion in a solution of

thiophanate heated to 70°C. With this treatment, seed germination was reduced by 36% and vigor was reduced by 38%.

2.2 INTRODUCTION

Fusarium wilt of cotton, caused by the soilborne fungus *Fusarium oxysporum* Schlechtend.:Fr f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans (FOV), is an increasingly important disease of cotton in the United States. Fusarium wilt occurs in all domesticated cotton (8). The fungus infects plants through the epidermal tissues of the root, invades and colonizes the vascular system, and grows and produces spores in xylem vessels. Thus, the fungus disrupts the plant's ability to transport nutrients and water. FOV cannot be easily eradicated from soil because it can colonize the roots of other crops and weeds and persist as chlamydospores (6, 19, 20, 21). Although the mechanism of seed infection is not clearly understood, the fungus colonizes the seed coat (8). This is of great concern for growers because of the possibility of dissemination of infected seed, especially with virulent isolates such as FOV race 4.

In the United States, FOV races 1, 2, 3, 4, and 8, as well as many un-named genotypes are present, but FOV race 4 is apparently restricted to California (3, 4, 5, 15, 23). Fusarium wilt caused by FOV race 4 has emerged as the most important disease concern for cotton growers because unlike other races and genotypes, race 4 can cause severe symptoms and economic losses in cotton in the absence of the root-knot nematode (*Meloidogyne* spp.) (6, 14, 15, 21, 22). In addition to FOV race 4, two virulent genotypes of FOV in Australia also cause severe symptoms on cotton in the absence of root-knot nematodes (9, 13). Currently, most cotton cultivars lack resistance to FOV race 4 (21). Since its discovery in California in 2001, race 4 has been confirmed in an increasing number of fields due primarily to seedborne dissemination (3, 4, 16). Although seed infection of FOV race 4 was reported to occur at very low frequencies (up to 0.015%), introduction of the fungus into a field is possible because over 100,000 cotton seeds are planted per hectare (1, 4). Because both cotton fiber and seed are produced in the same area in

California, the potential for spread of FOV race 4 by seed is high. The ability of FOV to persist in seed and survive for decades in soil are important considerations in the movement of seed from one area to another (6, 19, 20, 21). Currently, no available seed treatments can eliminate FOV from infected seed.

Thermotherapy alone or in conjunction with chemicals has been used as an effective seed treatment for various pathogens. In one study, *Fusarium moniliforme* was eliminated from infected maize seed when seed was pretreated in distilled water for 4 hours at 22°C and then immersed in a hot water bath for 5 min at 60°C (7). In another study, Fahim et al. found that a hot water treatment for 10-20 min at 60°C followed by a fungicide dressing eliminated *F. moniliforme* in maize seed without affecting seed germination (11). Moreover, the incidence of *F. circinatum* in infected *Pinus radiata* seed was significantly reduced when seed was immersed in a hot water bath for 30 min at 51–52°C (2). The same result was observed in gladiolus corms infected with *Fusarium oxysporum* f. sp. *gladioli* when cormels were immersed in hot water for 30 min at 53-55 °C (18). du Toit and Hernandez-Perez found that chlorine or hot water seed treatments substantially reduced or eliminated *Cladosporium variabile*, *Stemphylium botryosum*, and *Verticillium dahliae* in spinach seed without damaging seed germination (10). However, published research on the use of thermotherapy as a means to reduce the incidence of FOV in cotton seed is still limited.

To date, only one study has evaluated dry heat and hot water treatments as a means to eliminate FOV from cotton seed. Bennett and Colyer reported that seedborne FOV was reduced when seed was incubated in dry heat at 60, 70, and 80°C for 2 to 14 days, or immersed in 90°C water from 45 seconds to 3 min (3). The study reported here was conducted to further investigate the potential of thermotherapy as a means to eradicate FOV race 4 from infected seeds. The aim

of this study was to evaluate the efficacy of hot water treatments alone or in conjunction with fungicides and other treatments to reduce the viability of FOV race 4 in infected cotton seed.

2.3 MATERIALS AND METHODS

Culture maintenance and inoculum preparation. A monoconidial isolate of FOV race 4, isolate RBH1, was cultured on 9 cm-diameter petri plates containing acidified potato dextrose agar (APDA) under constant fluorescent light for 7 days at room temperature (22 ± 1°C). FOV race 4 RBH1 was originally isolated from a cotton plant in Kern County and confirmed to be race 4 by greenhouse pathogenicity tests and polymerase chain reaction (PCR) amplification of DNA using race 4-specific primers, R4f (5'GCTCCGTGTCWGAGCTTCTT) and R4r (5'GTTATGCTCCACGATGAG-CA) (23). Conidial suspensions were prepared from 1-wk-old APDA plates by flooding with 15 mL autoclaved deionized water, dislodging the conidia with a glass slide and filtering the suspension through four layers of sterile cheesecloth. The concentration of the conidial suspension was quantified using an Improved Neubauer hemacytometer (Hausser Scientific, Horsham, PA), and adjusted to $1x10^3$ and $1x10^4$ conidia per ml with sterile deionized water. The conidial suspensions were used to inoculate cotton bolls to produce infected seeds.

Inoculation of cotton bolls. In 2012 and 2013, two commercial fields of Acala cultivar PHY 725 RF *(*Dow AgroSciences, Midland, MI*)* in Los Banos, California were used to generate seed infected with FOV race 4 isolate RBH1. Two sites in each field were used each year. Immature and unopened bolls, at least 2.5 cm in diameter, on cotton plants with 16 to 17 mainstem nodes were injected 1 to 3 times between each seam of the carpel wall with a syringe and needle containing approximately 5 to 10 µL of a 10^3 or 10^4 cfu/mL spore suspension. Water was used to

mock inoculate cotton bolls for seed that served as a negative control. Matured bolls were hand harvested and ginned. Ginned seeds were delinted in 93% sulfuric acid, rinsed with water, and neutralized with sodium bicarbonate. Seed were then dried at 35 to 38°C for 36 hours and stored at room temperature (22 to 23°C). The seed was stored for less than 1 month before use.

Seed germination and vigor assays. To determine the effect of hot water immersion on seed quality, seed germination and vigor assays were conducted on both Upland and Pima cultivars following AOSA guidelines. A sample of 200 seed from each seed lot was assayed for germination and vigor on several 25.4 × 38 cm sheets of heavy weight germination paper (Anchor Paper Company, St. Paul, MN) as describe by Bennett and Colyer (3). Vigor was defined as the percentage of those seedlings that had a combination of hypocotyl length and root length that exceeded 4 cm following incubation of the seed at 18°C for 7 days as described by Savoy (17). Cottonseed of Acala cultivar PHY 72, Upland cultivar PHY 98M-2983, and Pima cultivar PHY 800 from commercial seedlots harvested from fields free of FOV in 2011 were immersed in a 2 L beaker of distilled water which was heated in a 10 L water bath. Treatments included immersion in a hot water bath for 20 min at 55°C, 10 min at 38°C followed by 20 min at 55°C, 20 min at 60°C, 20 min at 65°C, 20 min at 70°C, 10 min at 38°C followed by 20 min at 70°C, 30 min at 60°C, 40 min at 60°C, 105 seconds at 90°C, or 10 min for 38°C followed by 105 seconds 90°C. All seed was then cooled for 5 min in 4 L of 22°C distilled water followed by 3 days at ambient room temperature in a seed dryer or laminar flow hood. All treatments were replicated three times and the experiment was repeated once.

Hot water treatments, 2012. The effect of hot water on the incidence of FOV in seed from the artificially inoculated bolls was evaluated. For each temperature and time combination, each of three nylon bags containing 400 seed was immersed in a 2 L beaker of distilled water heated to

55 or 60℃ (±0.05°C) in a 10 L water bath. Water temperature was constantly monitored with a mercury thermometer. Seeds were immersed for 20 min at 23°C (control), 20 min at 55°C, 10 min at 38°C followed by 20 min at 55°C, or 20 min at 60°C. After each temperature–time combination, seeds were removed from the water bath and immediately immersed for 5 min in 4 L of distilled water maintained at 22°C. The seed was dried for 3 days in a seed dryer or laminar flow hood at ambient room temperature. After drying, the seed was immersed in a 10% solution of household bleach (0.6% NaOCl, w/v) for 1 minute to eliminate surface contaminants. All 400 seeds in each treatment were plated on Komada's medium (about 12 seeds per plate). Plates were monitored at 7 and 14 days for growth of FOV. A random sub-sample of 50 potential FOV colonies from all treatments was transferred onto APDA and incubated under constant fluorescent light at room temperature (22 ± 1°C) to verify the identity of the fungus. After 5 days, DNA was extracted from *Fusarium*-like colonies using Qiagen® DNeasy Plant Mini Kit ™ (Valencia, CA) according to the manufacturer's protocol. FOV race 4 isolates were identified by amplifying a unique sequence of DNA using race 4-specific primers (23). To determine the percentage of infection in the seed before treatment, 400 nontreated seeds were plated, monitored, and isolates of FOV race 4 were detected as previously described. The experiment was repeated once for each set of seeds.

Seed treatments 2013, trial 1. Various treatments were evaluated on seed (cv. PHY 725-RF) harvested in 2013. For each treatment, three nylon bags containing 400 seeds each were pretreated at 24°C and agitated at 100 rpm for 1 hour in 1 L of sterile deionized water or sterile 30% potato dextrose broth (PDB). In addition, three nylon bags of 400 seeds each were left untreated. After pretreatment, seed (including untreated seed) was immersed for 20 min at 60°C in 2 L of the following solutions: sterile deionized water, 10% solution of household bleach

(0.6% NaOCl, w/v), 0.5% sodium lauryl sulfate (SLS), 0.25 g a.i. of fludioxonil (*Cannonball*® WP) per liter of water, 0.211 g a.i. of thiabendazole (*Mertect*® 340-F) per liter of water, 0.12 g a.i. of azoxystrobin (*Quadris*® Flowable) per liter of water, 0.35 g a.i. of thiophanate (*Topsin*® M 70WP) per liter of water, or a fungicide bath containing a combination of 0.12 g a.i. of azoxystrobin , 0.25 g a.i. of fludioxonil, 0.211 g a.i. of thiabendazole, and 0.35 g a.i. of thiophanate per liter of water. After each pretreatment-treatment combination, all seed was immediately immersed for 5 min in 4 L of 22°C distilled water, dried for 3 days, plated, monitored, and isolates of FOV race 4 were detected as previously described. To determine the percentage of infection in the seed prior to the treatments, 400 nontreated seeds were surface sterilized in 10% bleach for 1 minute and plated on Komada's medium (about 12 seeds per plate). Germination and vigor assays were conducted on seed from all pretreatment and treatment combinations as previously described.

Greenhouse grow-out. Seed of some of the treatments were sown in 128 cell-seedling trays (100 seeds per tray) with 0.06 cubic centimeter cells containing autoclaved UC potting soil mix (12). A total of 600 seed was distributed evenly in six trays arranged in a completely randomized block design in the greenhouse on a 13-h photoperiod provided by high pressure sodium bulbs with daytime temperatures ranging from 85-90°C and nighttime temperatures ranging from 65-70°C. The incidence of FOV in seed was assessed by quantifying the number of seedlings with symptoms typical of Fusarium wilt (wilting, dark brown vascular discoloration, and interveinal and marginal leaf chlorosis, necrosis, and death). The experiment was repeated twice for each set of seed from the two sites in the commercial field in 2013. Roots from a random sub-sample of 50 symptomatic plants from all treatments were washed with anti-bacterial soap, immersed 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, and FOV race 4 isolates were identified with PCR using race 4-specific primers as previously described.

Seed treatments 2013, trial 2. In an attempt to eradicate FOV from seed, additional hot water treatments were evaluated on infected seed of Acala *P*HY 725-RF from the 2013 harvest of inoculated bolls. For each treatment, 400 seeds were either pretreated or not pretreated at 24°C and agitated at 100 rpm for 1 hour in 1 L of sterile deionized water. The seed was then immersed for 20 min at 70, 75, or 80 °C in 2L of water or thiophanate (0.35 g a.i. /L water). After each pretreatment-treatment combination, the seed was immediately immersed for 5 min in 4L of distilled water maintained at 22°C, dried for 3 days, plated and monitored. Isolates of FOV race 4 were detected as previously described. To determine the percentage of infected seed before treatment, 400 nontreated seeds were plated, monitored, and isolates of FOV race 4 were detected as previously described. The experiment was repeated twice for each set of seed from the two sites in the commercial field in 2013. Seed germination and vigor assays were conducted on all pretreatment-treatment combinations as previously described.

Data analyses. Data from all experiments were analyzed using the Statistical Analysis Software (SAS) 9.3 general linear model. Significance of pretreatment*treatments and their interaction on the incidence of FOV race 4 in infected seed was tested using analysis of variance (ANOVA). Where significant F values were obtained, the significance of mean differences was assessed using Tukey's HSD test.

2.4 RESULTS

Seed germination and vigor assays. There was no significant difference in seed germination and seedling vigor between the two independent trials; therefore, data from the two trials were analyzed together. Seed germination and vigor were not affected by hot water treatments for 20 min at 55°C, 10 min at 38°C followed by 20 min at 55°C (data not shown), or 20 min at 60°C (Figure 2.1). Treatments for 20 min at 65 or 70°C significantly reduced both germination and vigor (Figure 2.1), as well as treatments of 10 min at 38°C followed by 20 min at 70°C, 30 min at 60°C, 40 min at 60°C, 105 seconds at 90°C, and 10 min at 38°C followed by 105 seconds at 90°C (data not shown).

Hot water treatments, 2012. The incidence of FOV race 4 in seed from inoculated bolls from sites 1 and 2 was 10.5% and 10.0%, respectively. Data from two trials were analyzed separately because the experiment*treatment interaction was significant. There was a significant interaction in the incidence of seed infection between the sites and the treatments (P=0.02). The incidence of seed infection was significantly reduced when seed was immersed in water for 20 min at 55°C, 10 min at 38°C followed by 20 min at 55°C, or 20 min at 60°C (Figure 2.2). There was no significant difference in seed infection when seed was immersed for 20 min at 55°C or 10 min at 38°C followed by 20 min at 55°C (data not shown). FOV race 4 was re-isolated and confirmed in all of the 50 random sub-samples of FOV colonies transferred to APDA.

Seed treatments 2013, trial 1. Following inoculation of bolls in 2013, harvested seed was frequently infected with FOV race 4. Prior to the treatments, the incidence of FOV seed from inoculated bolls from sites 1 and 2 was 40.2% and 37.4%, respectively. There was a significant interaction in the incidence of seed infection between the sites and the treatments (P=0.01). The

combination of PDB pretreatment followed by a 20 minute immersion in a 60°C fungicide bath containing all four fungicides was one of the best pretreatment-treatment combinations. This treatment reduced the incidence of FOV in seed from 40.2% to 5.5% in seed from site 1 (Table 1) and from 37.4% to 5.6% in seed from site 2 (Table 2). Among the other best pretreatment-treatment combinations for reducing the incidence of FOV in seed in both experimental sites were the PDB pretreatment followed by thiabendazole, thiophanate, or the combination of fungicides and the water pretreatment followed by thiophanate or the combination of fungicides (Tables 1 and 2). There was no significant difference in the incidence of infected seed among these pretreatment-treatment combinations. Among the least effective pretreatment-treatment combinations for reducing FOV from seed were the nontreated pretreatment followed by water, azoxystrobin, or bleach. In general, the PDB and water pretreatments were significantly more effective in reducing FOV from seed than the nontreated pretreatment. Germination and vigor of seed from both field sites were not reduced with the exception of SLS treatments where germination and vigor were reduced by 26.2% (data not shown). FOV race 4 was re-isolated and confirmed in all of the 50 random sub-samples of FOV colonies transferred to APDA.

Greenhouse grow-out. In the greenhouse grow-out experiments, seedlings grown from nontreated seeds from site 1 and 2 in 2013 developed symptoms typical of Fusarium wilt in approximately 39.6% (Table 3) and 37.1% (Table 4) of the plants, respectively. Approximately 0.3% of seed harvested from bolls that were mock-inoculated with water in both field sites did not germinate. The PDB pretreatment followed by a 20 minute immersion in the combination of fungicides or thiophanate at 60°C significantly reduced FOV in seed (Tables 1, 2, 3, 4). Based on infected seedlings, the PDB pretreatment was more effective in reducing the incidence of FOV in seed than the nontreated pretreatment. Immersion of seed for 20 min in SLS at 60°C reduced

incidence of FOV whether the seed was presoaked in PDB or not. However, germination was reduced by about 16 % (data not shown). The pretreatment-treatment combinations of PDB followed by thiophanate or all fungicides significantly reduced the incidence of wilt symptoms by approximately 80% and 85%, respectively. These were among the best pretreatment-treatment combinations. There was no significant difference in the incidence of infected seed between these pretreatment-treatment combinations. When seed was pretreated with PDB or not pretreated followed by a 20 minute immersion in SLS at 60°C, the incidence of FOV in site 2 was reduced by approximately 75% and 62%, respectively. However a reduction in seed germination of 15.7% was also observed (data not shown). FOV race 4 was re-isolated from all 50 of the randomly sampled symptomatic plants.

Seed treatments 2013, trial 2. FOV race 4 was eradicated from seed from both sites in 2013 when seeds were immersed in water at 24°C followed by immersion for 20 min in the thiophanate solution at 70°C, thiophanate solution at 75°C, thiophanate solution at 80°C, or water at 80°C. In addition, FOV was eliminated from seed immersed for 20 min in a thiophanate solution at 80°C (Tables 5 and 6). FOV was not eliminated from seed that was not pretreated, whether followed by 20 min immersion in hot water at 70°C, thiophanate slurry at 70°C, water at 75°C, or thiophanate slurry at 75°C.

Seed germination was reduced by 36% and vigor was reduced by 38% by the pretreatment-treatment combinations of water or untreated pretreatment followed by immersion in water or thiophanate slurry at 70°C for 20 min. In pretreatment-treatment combinations where seed was immersed for 20 min at 75°C, seed germination was reduced by 59% and vigor was reduced by 74%. Seed germination was reduced by 95% and vigor was reduced by 98% when seed was immersed for 20 min at 80°C (Figure 2.3).

2.5 DISCUSSION

Infested seed can serve as a vehicle for movement of FOV into fields or regions not previously affected by Fusarium wilt. The need for an effective seed treatment to prevent this mode of dispersal is underscored by the emergence of new pathotypes such as race 4. Bennett and Colyer found that the incidence of FOV in infected seed was reduced by 80% when seed was immersed for 105 seconds in water heated to 90°C (3). They also found that this reduction of FOV was achieved without a significant reduction in seed germination or vigor. However, our results showed otherwise. Seed germination was reduced by 42.4% and vigor was reduced by 55% when seed was immersed for 105 seconds at 90°C. In our experiments, germination and vigor were not affected by hot water treatments at 60°C for 20 min but were negatively affected at higher temperatures. The incidence of FOV race 4 in seed was reduced by approximately 56% when infected seed was immersed in water maintained at 60°C for 20 min. The disparity between our results and those of Bennett and Colyer may be attributed by the use of different seedlots, age of seed, or other unknown factors.

In order to improve the efficacy of hot water seed treatments, seed was pretreated with PDB or water followed by chemical treatments. The PDB or water pretreatment enhanced the effectiveness of the chemical treatments. For example, when seed was simply immersed in a 60°C bath of a combination of fungicides for 20 min, the incidence of FOV was reduced by approximately 76%, but when seed was pretreated with water at 24°C for 1 hour followed by immersion in a bath of a combination of fungicides, the incidence of FOV in seed was reduced by approximately 84%. Overall, the water pretreatment was significantly better at reducing the incidence of FOV in seed than no pretreatment. In addition, when seed was pretreated in PDB at 24°C and immersed for 20 min in a combination of fungicides solution at 60°C, FOV in seed

was reduced by approximately 86% based on recovery on cultures and greenhouse assays. The PDB pretreatment was significantly better at reducing the incidence of FOV in seed than no pretreatment. However, the PDB pretreatment compared to water pretreatment did not increase the efficacy of the 60°C bath of combination of fungicides. The mechanism for the increased effectiveness of the chemical seed treatment when a pretreatment is used is not known. Perhaps the pretreatments make the seed coat more permeable to chemical treatment. Another possibility is that pretreatments induce germination of FOV conidia or chlamydospores, making them more susceptible to chemical and heat treatments.

The most effective treatments evaluated here are pretreatment-treatment combinations of a 1 hour immersion in water or PDB maintained at 24°C followed by immersion for 20 min in a solution of thiophanate or combination of fungicides heated to 60°C. Combining water or PDB pretreatments with thiophanate or a combination of fungicides reduced seed infection to a greater extent than when fungicides were used alone. Water pretreatment followed by an immersion in thiophanate or a combination of fungicides was as effective in reducing the incidence of FOV in seed as PDB pretreatment followed by an immersion in thiophanate or a combination of fungicides. The incidence of FOV in the greenhouse grow-out assay almost paralleled the recovery of FOV from the seed in the plate assay, indicating that a seed infection almost always resulted in an infected plant in the greenhouse grow-out. Since both cotton fiber and seed are produced in the same area in California, this demonstrates an efficient method of dissemination of FOV in California.

Thiophanate alone was as effective as a combination of fungicides. The economical choice for industry is to pretreat the seeds with water followed by immersion for 20 min in thiophanate heated to 60°C. Although FOV race 4 was eliminated from infected seed when seed

was pretreated for 1 hour in water followed by 20 min in a 70°C thiophanate bath, seed germination was reduced by 36% while vigor was reduced by 38%. FOV race 4 was not eliminated from infected seed when seed was pretreated for 1 hour in water followed by 20 min in a 60°C thiophanate bath. Instead, the incidence of FOV was reduced by ~80% without a reduction in seed germination and vigor. The ability to eliminate FOV from seed may have limited but important applications for moving seed from one location to another, such as in breeding trials.

The pretreatment with water or PDB is necessary and recommended because these measures significantly reduced incidence of FOV race 4 in infested seed. Although the seed treatments mentioned here can be implemented for commercial use, further research is required to find a combination of chemical, temperature and time that eliminates FOV from seed without significantly reducing seed germination and vigor. The results here were based on seed that was infested through an artificial procedure. Naturally infested seed may respond differently to seed treatments.

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Table 1. Effect of immersing cotton seed in water or potato dextrose broth followed by immersion in fungicides or other treatments on viability of *Fusarium oxysporum* f. sp. *vasinfectum* in seed, 2013, site 1.

Pretreatment (24°C, 100rpm, 1 hr)	**Treatment (60°C, 20min)**	**Rate (g a.i./L water)**	**Infection (%)[W]**
Nontreated	Nontreated	0	40.2 a[X]
Nontreated	Water	0	19.2 b
Nontreated	Bleach	0.006	15.9 cd
Nontreated	Sodium lauryl sulfate	5	15.7 cd
Nontreated	Azoxystrobin	0.12	16.3 bc
Nontreated	Fludioxonil	0.25	14.3 cde
Nontreated	Thiabendazole	0.211	13.4 cdef
Nontreated	Thiophanate	0.35	10.3 fgh
Nontreated	All fungicides	--[Y]	9.7 ghi
Water	Nontreated	0	39.7 a
Water	Water	0	15.7 cd
Water	Bleach	0.006	14.5 cde
Water	Sodium lauryl sulfate	5	14.3 cde
Water	Azoxystrobin	0.12	14.0 cde
Water	Fludioxonil	0.25	12.9 defg
Water	Thiabendazole	0.211	9.2 hij
Water	Thiophanate	0.35	7.9 ijk
Water	All fungicides	--	6.4 jk
Potato dextrose broth[Z]	Nontreated	0	39.9 a
Potato dextrose broth	Water	0	14.1 cde
Potato dextrose broth	Bleach	0.006	14.5 cde
Potato dextrose broth	Sodium lauryl sulfate	5	13.1 def
Potato dextrose broth	Azoxystrobin	0.12	13.5 cde
Potato dextrose broth	Fludioxonil	0.25	12.3 efgh
Potato dextrose broth	Thiabendazole	0.211	8.8 ij
Potato dextrose broth	Thiophanate	0.35	6.2 jk
Potato dextrose broth	All fungicides	--	5.5 k

[W]Infection (%) is based on recovery of *F. oxysporum* f. sp. *vasinfectum* on Komada's medium.
[X]Means of infections (%) followed by a common letter are not significantly different according to Tukey's HSD at $P = 0.05$. Values are means of six replications.
[Y]All fungicides = 0.12 g a.i. of azoxystrobin, 0.25 g a.i. of fludioxonil, 0.211 g a.i. of thiabendazole, and 0.35 g a.i. of thiophanate per liter of water.
[Z]30% potato dextrose broth (PDB) composed of 1.2 grams potato starch and 6 grams of dextrose per liter of water.

Table 2. Effect of immersing cotton seed in water or potato dextrose broth followed by immersion in fungicides or other treatments on viability of *Fusarium oxysporum* f. sp. *vasinfectum* in seed, 2013, site 2.

Pretreatment (24°C, 100rpm, 1 hr)	Treatment (60°C, 20min)	Rate (g a.i./L water)	Infection (%)[W]
Nontreated	Nontreated	0	37.4 a[X]
Nontreated	Water	0	18.2 b
Nontreated	Bleach	0.006	15.0 cd
Nontreated	Sodium lauryl sulfate	5	14.9 cd
Nontreated	Azoxystrobin	0.12	16.0 bc
Nontreated	Fludioxonil	0.25	14.3 cd
Nontreated	Thiabendazole	0.211	13.1 cd
Nontreated	Thiophanate	0.35	9.9 e
Nontreated	All fungicides	--[Y]	9.1 fg
Water	Nontreated	0	37.3 a
Water	Water	0	15.5 bcd
Water	Bleach	0.006	14.2 cd
Water	Sodium lauryl sulfate	5	13.8 cd
Water	Azoxystrobin	0.12	13.5 cd
Water	Fludioxonil	0.25	13.1 cd
Water	Thiabendazole	0.211	8.1 fg
Water	Thiophanate	0.35	7.7 fgh
Water	All fungicides	--	6.0 gh
Potato dextrose broth[Z]	Nontreated	0.006	37.1 a
Potato dextrose broth	Water	0.12	13.8 cd
Potato dextrose broth	Bleach	0	14.0 cd
Potato dextrose broth	Sodium lauryl sulfate	0.25	13.1 cd
Potato dextrose broth	Azoxystrobin	5	13.4 cd
Potato dextrose broth	Fludioxonil	0.211	12.1 de
Potato dextrose broth	Thiabendazole	0.35	6.6 gh
Potato dextrose broth	Thiophanate	--	6.3 gh
Potato dextrose broth	All fungicides	0	5.6 h

[W]Infection (%) is based on recovery of *F. oxysporum* f. sp. *vasinfectum* on Komada's medium.

[X]Means of infections (%) followed by a common letter are not significantly different according to Tukey's HSD at $P = 0.05$. Values are means of six replications.

[Y]All fungicides = 0.12 g a.i. of azoxystrobin, 0.25 g a.i. of fludioxonil, 0.211 g a.i. of thiabendazole, and 0.35 g a.i. of thiophanate per liter of water.

[Y]30% potato dextrose broth (PDB) composed of 1.2 grams potato starch and 6 grams of dextrose per liter of water.

Table 3. Effect of immersing cotton seed in potato dextrose broth followed by immersion in fungicides or other treatments on the incidence of *Fusarium oxysporum* f. sp. *vasinfectum* in seedlings in a greenhouse grow-out assay, 2013, site 1.

Pretreatment (24°C,100rpm, 1 hr)	**Treatment (60°C, 20min)**	**Rate (g a.i./L water)**	**Infection (%)[V]**
Nontreated	Nontreated	0	39.6 a[W]
Nontreated	Water	0	19.3 b
Nontreated	Sodium lauryl sulfate	5	15.3 c
Nontreated	Thiophanate	0.35	10.3 e
Nontreated	All fungicides	--[X]	9.3 e
Nontreated	Noninoculated[Y]	0	0.3 g
Potato dextrose broth[Z]	Nontreated	0	39.6 a
Potato dextrose broth	Water	0	14.5 c
Potato dextrose broth	Sodium lauryl sulfate	5	12.5 d
Potato dextrose broth	Thiophanate	0.35	5.8 f
Potato dextrose broth	All fungicides	--	5.6 f
Potato dextrose broth	Noninoculated	0	0.3 g

[V] Infection (%) is based on recovery of *F. oxysporum* f. sp. *vasinfectum* on Komada's medium.
[W]Means of infections (%) followed by a common letter are not significantly different according to Tukey's HSD at $P = 0.05$. Values are means of six replications.
[X]All fungicides =0.12 g a.i. of azoxystrobin, 0.25 g a.i. of fludioxonil, 0.211 g a.i. of thiabendazole, and 0.35 g a.i. of thiophanate per liter of water.
[Y] Seed harvested from mock (water) inoculated cotton bolls.
[Z] 30% potato dextrose broth (PDB) composed of 1.2 grams potato starch and 6 grams of dextrose per liter of water.

Table 4. Effect of immersing cotton seed in potato dextrose broth followed by immersion in fungicides or other treatments on the incidence of *Fusarium oxysporum* f. sp. *vasinfectum* in seedlings in a greenhouse grow-out assay, 2013, site 2.

Pretreatment (24°C,100rpm, 1 hr)	**Treatment (60°C, 20min)**	**Rate (g a.i./L water)**	**Infection (%)[V]**
Nontreated	Nontreated	0	37.1 a[W]
Nontreated	Water	0	18.4 b
Nontreated	Sodium lauryl sulfate	5	14.5 c
Nontreated	Thiophanate	0.35	9.9 e
Nontreated	All fungicide	--[X]	9.4 e
Nontreated	Noninoculated [Y]	0	0.3 g
Potato dextrose broth[Z]	Nontreated	0	36.8 a
Potato dextrose broth	Water	0	13.3 d
Potato dextrose broth	Sodium lauryl sulfate	5	12.5 d
Potato dextrose broth	Thiophanate	0.35	6.3 f
Potato dextrose broth	All fungicide	--	5.4 f
Potato dextrose broth	Noninoculated	0	0.3 g

[V] Infection (%) is based on recovery of *F. oxysporum* f. sp. *vasinfectum* on Komada's medium.
[W]Means of infections (%) followed by a common letter are not significantly different according to Tukey's HSD at P = 0.05. Values are means of six replications.
[X]All fungicides =0.12 g a.i. of azoxystrobin, 0.25 g a.i. of fludioxonil, 0.211 g a.i. of thiabendazole, and 0.35 g a.i. of thiophanate per liter of water.
[Y] Seed harvested from mock (water) inoculated cotton bolls.
[Z] 30% potato dextrose broth (PDB) composed of 1.2 grams potato starch and 6 grams of dextrose per liter of water.

Table 5. Effect of immersion temperature in water or a fungicide on viability of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton seed, 2013, site 1.

Pretreatment (24°C, 100rpm, 1 hr)	**Treatment (60°C, 20min)**	**Rate (g a.i./L water)**	**Infection (%)[V]**
Nontreated	Nontreated	0	39.7 a[W]
Nontreated	Water, 70°C	0	1.6 b
Nontreated	Thiophanate, 70°C	0.35	0.7 bc
Nontreated	Water 75°C	0	1.0 bc
Nontreated	Thiophanate, 75°C	0.35	0.3 bc
Nontreated	Water 80°C	0	0.4 bc
Nontreated	Thiophanate, 80°C	0.35	0 c
Water	Nontreated	0	39.7 a
Water	Water, 70°C	0	1.3 bc
Water	Thiophanate, 70°C	0.35	0 c
Water	Water 75°C	0	0.3 bc
Water	Thiophanate, 75°C	0.35	0 c
Water	Water 80°C	0	0 c
Water	Thiophanate, 80°C	0.35	0 c

[V] Infection (%) is based on recovery of *F. oxysporum* f. sp. *vasinfectum* on Komada's medium.
[W]Means of infections (%) followed by a common letter are not significantly different according to Tukey's HSD at P = 0.05. Values are means of six replications.

Table 6. Effect of immersion temperature in water or a fungicide on viability of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton seed, 2013, site 2.

Pretreatment (24°C, 100rpm, 1 hr)	**Treatment (60°C, 20min)**	**Rate at Treating (g a.i./L water)**	**Infection (%)[V]**
Nontreated	Nontreated	0	37.6 a[W]
Nontreated	Water, 70°C	0	1.5 b
Nontreated	Thiophanate, 70°C	0.35	0.6 cde
Nontreated	Water 75°C	0	0.9 bcd
Nontreated	Thiophanate, 75°C	0.35	0.2 de
Nontreated	Water 80°C	0	0.5 cde
Nontreated	Thiophanate, 80°C	0.35	0 d
water	Nontreated	0	36.9 a
water	Water, 70°C	0	1.3 bc
water	Thiophanate, 70°C	0.35	0 d
water	Water 75°C	0	0.2 de
water	Thiophanate, 75°C	0.35	0 d
water	Water 80°C	0	0 d
water	Thiophanate, 80°C	0.35	0 d

[V] Infection (%) is based on recovery of *F. oxysporum* f. sp. *vasinfectum* on Komada's medium.
[W]Means of infections (%) followed by a common letter are not significantly different according to Tukey's HSD at $P = 0.05$. Values are means of six replications.

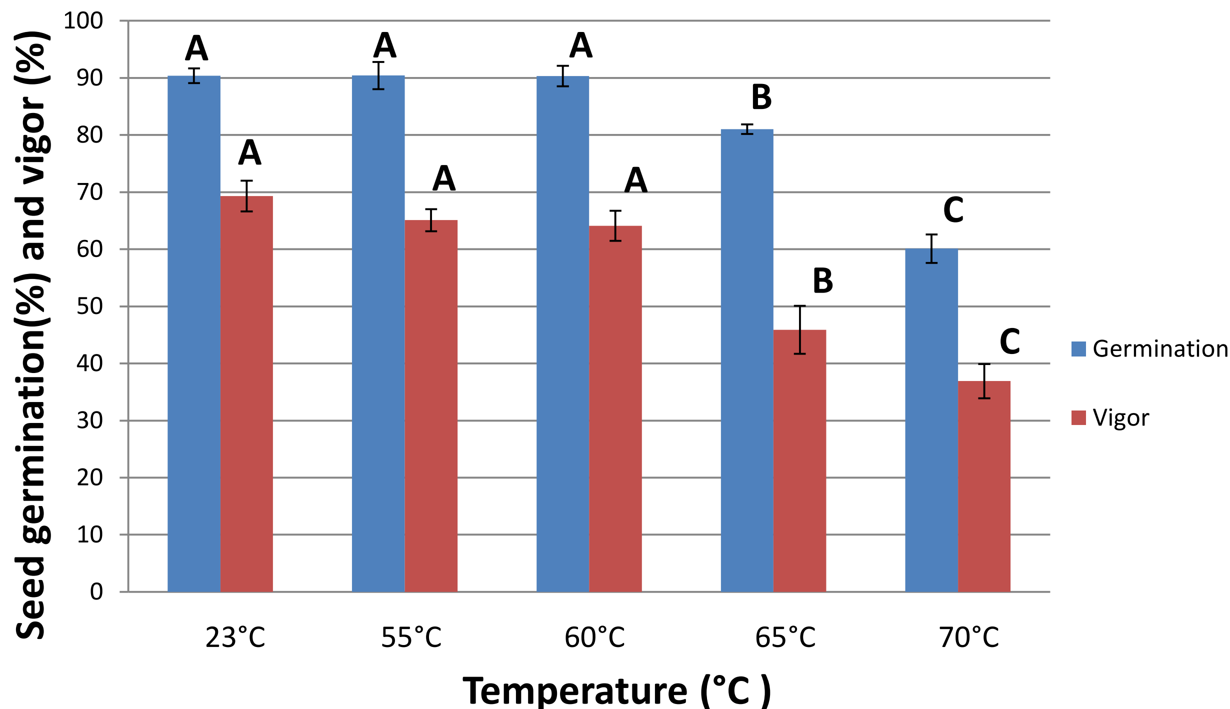

Figure 2.1. Effect of hot water treatments on germination and vigor of cotton seeds, 2012.Values represent average percentages of germination and vigor of cotton seed (PHY 830, PHY 2983, PHY 72) of three replications in each of two independent trials. Germination and vigor assays were conducted following AOSA guidelines. Two hundred seed of each cultivar was immersed for 20 min in a hot water bath heated to 55, 60, 65, or 70°C. Germination and vigor are independent variables. Means are separated independently. Vigor was defined as the percentage of those seedlings that had a combination of hypocotyl length and root length that exceeded 4 cm following germination of the seed at 18°C for 7 days. Different letters for germination or vigor indicate significant differences according to Tukey's HSD at $P < 0.05$.

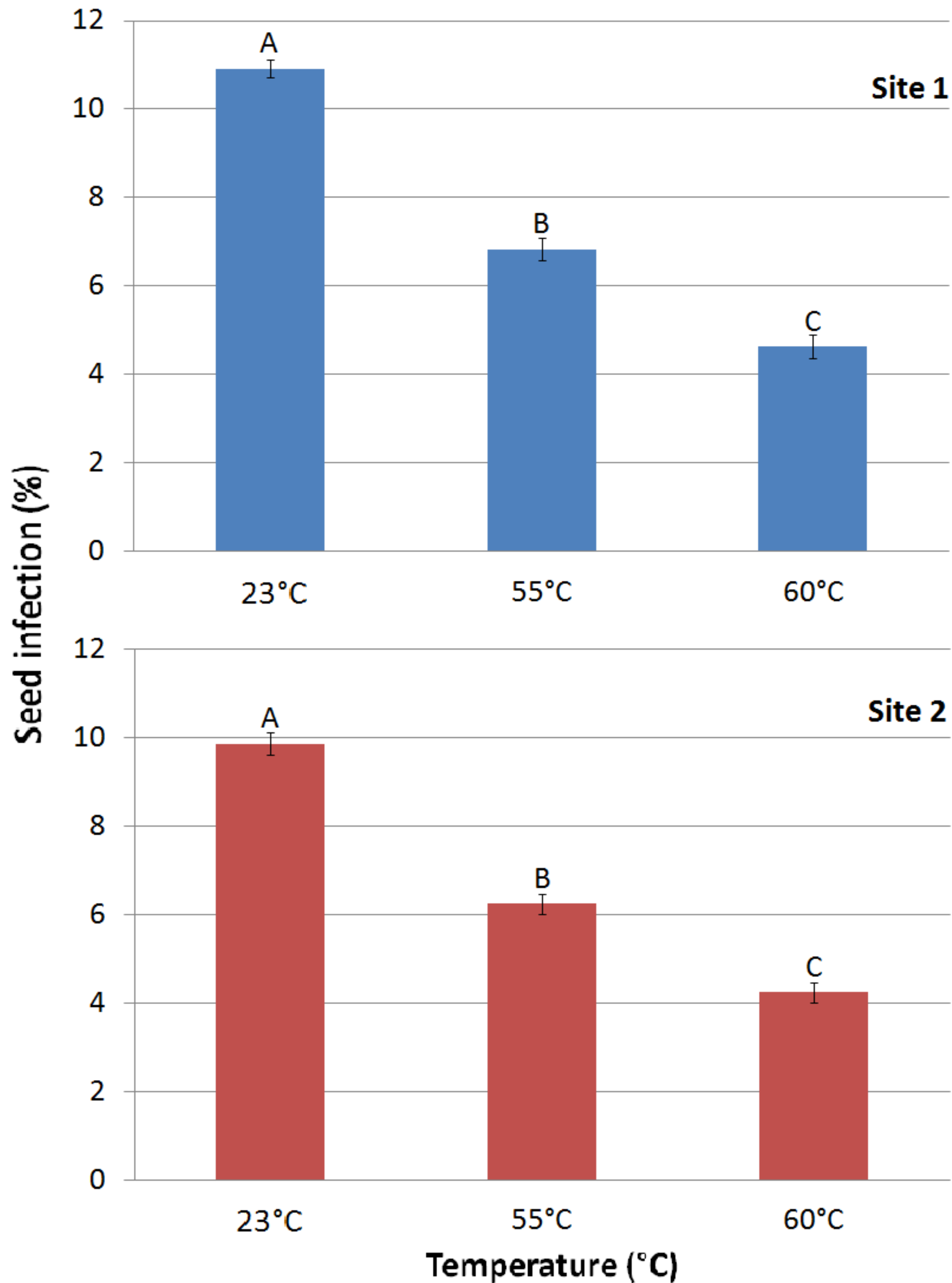

Figure 2.2. Effect of hot water treatments on the incidence of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 in cotton seed, 2012. Values represent average percentages of infected seed (PHY 725 RF) harvested from two sites in a commercial field, 2012. There were three replications for each treatment in each trial. Two hundred seed of each cultivar was immersed for 20 min in a hot water bath heated to 55 or 60°C. Error bars are standard deviations. Different letters indicate significant differences according to Tukey's HSD at $P < 0.05$. Seed infection was based on recovery of *F. oxysporum* f. sp. *vasinfectum* on Komada's medium.

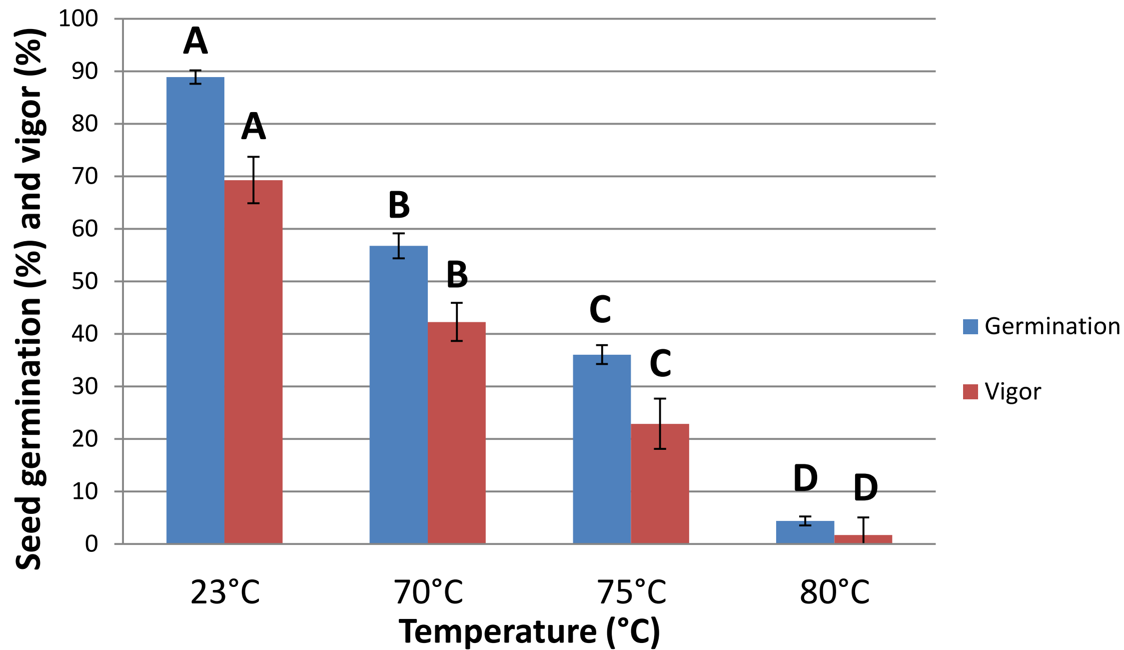

Figure 2.3. Effect of hot water treatments on germination and vigor of cotton seeds, 2013. Values represent average percentages of germination and vigor of cotton seed (PHY 830, PHY 2983, PHY 72) of three replications in each of two independent trials. Two hundred seed of each cultivar was immersed for 20 min in a hot water bath heated to 70, 75, or 80°C. Germination and vigor are independent variables. Means are separated independently. Vigor was defined as the percentage of those seedlings that had a combination of hypocotyl length and root length that exceeded 4 cm following germination of the seed at 18°C for 7 days.

CHAPTER III

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

3.1 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.

3.2 INTRODUCTION

Fusarium wilt of cotton, caused by the soilborne fungus *Fusarium oxysporum* Schlechtend.:Fr f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans, is a widespread disease occurring in most cotton-growing areas of the world. The disease was first described by Atkinson in the U.S. in 1892 (5). Currently, six races are recognized: 1, 2, 3, 4, 6, and 8, as well as many un-named genotypes worldwide (5, 9). Many are widespread in the U.S., but race 4 (FOVr4), which is highly virulent, is apparently restricted to California (1, 2, 3, 9, 18). Race 4 is of great concern to growers because most cotton cultivars lack resistance to it. Unlike other races, race 4 can cause severe symptoms in cotton, especially Pima cultivars (*Gossypium barbadense* L.), in the absence of the root-knot nematode (*Meloidogyne* spp.) (4, 8, 9, 16). FOVr4 is found in an increasing number of fields in California due in part to seed-borne dissemination (1, 2, 11). The disease can quickly become widespread as residue from infected plants release numerous propagules into the soil, potentially increasing disease severity in subsequent cotton crops. In addition, the disease is very difficult to manage and once a field becomes infested, there are no cost-effective means to eradicate the pathogen from the soil. *F. oxysporum* f. sp. *vasinfectum* (FOV) persists in the soil indefinitely because it can colonize the roots of other crops and weeds (4, 14, 15, 16). In addition, FOV overwinters as chlamydospores, which apparently survive in the soil for a number of years (14).

California produces over 90% of Pima cotton fiber and seed in the U.S. (17). Both fiber and seed are produced in the same area of the state; therefore, the potential for contamination of seed by FOV race 4 is high (2). Currently there are no effective 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’GCTCCGTGTCWGAGCTTCTT) and R4r (5’GTTATGCTCCACGATGAG-CA), is presently used to detect 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.

3.3 MATERIALS, METHODS AND RESULTS

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, Agdia). 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) (Figure 3.1). The whole detection apparatus was then assembled and the result was observed within 20 min.

3.3.1 Evaluation of AmplifyRP® Acceler8™ for FOVr4

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 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 density of spores was determined with a hemacytometer and adjusted to 1×10^7 conidia per ml by the addition of 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 ($1x10^7$ conidia per ml) from cultures of FOV races 1, 2, 3, 4, 6, 8, or water (non-infected control) and transplanted into plastic pots 10 cm in diameter (750ml) containing UC potting soil mix (7). Seedlings of Phytogen 800, a cotton cultivar resistant to FOV race 4, was also inoculated with a conidial suspension of FOV race 4. Four weeks post-inoculation, 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 directly applied to the AmplifyRP® Acceler8™ kit. Four plants infected with each race and the water control were assayed and the test was conducted three separate times on different plants.

The AmplifyRP® Acceler8™ assay detected FOVr4 (a testing line on the test strip indicated a positive reaction) in all FOV race 4 mycelial preparations, conidial preparations, and stem tissues (Figure 3.2).

Field Samples. Pima cotton plants (Phytogen 805RF) with symptoms typical of Fusarium wilt were collected from four FOVr4-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, immersed in 0.6% sodium hypochlorite (10% bleach) for 1 minute, 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 protocol. For the direct DNA extraction method, 300 mg fresh weight of taproot tissue with symptoms typical of Fusarium wilt was ground in liquid nitrogen with a mortar and pestle and DNA was extracted as before. DNA from cultures and roots was independently assayed with race 4 specific primers in conventional PCR reactions. Ten microliters of the PCR product was visualized by gel electrophoresis on a 1.5% agarose gel run at 100 volts for 25 min stained with ethidium bromide. Four plants 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 FOVr4 in Various Plant Tissues. Three hundred mg was collected from each of the following tissues from FOVr 4-inoculated and non-inoculated 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 (Figure 3.3). 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 main stem nodes 1 and 2 or main stem nodes 2 and 3. The fungus was never detected in 12 attempts in stem tissues without vascular discoloration between the main stem 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-treated 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 also was recovered by culture or detected by conventional PCR. In no case was race 4 detected in non-inoculated plants.

Inoculated Soil Samples. FOV race 4 conidial suspensions were prepared and adjusted to 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 conidia per 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 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 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 $1x10^7$, $1x10^6$, $1x10^5$, or $1x10^4$ conidia/gram of dried soil. In UCD potting soil inoculated with $1x10^3$ 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 $1x10^2$ or $1x10^1$ conidia/gram of dried soil. In field soil, the AmplifyRP® Acceler8™ kit detected FOV race 4 in all 12 soil samples amended with $1x10^7$, $1x10^6$, and $1x10^5$conidia/gram of dried soil. In field soil inoculated with $1x10^4$ 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 $1x10^3$, $1x10^2$, or $1x10^1$ 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 directly applied to the AmplifyRP® Acceler8™ kit. The same amount of soil was also assayed with conventional PCR using race 4 specific primers by the culture-based isolation method and through direct DNA extraction. For the culture-based isolation method, 300 mg of soil was suspended in a flask of 100 mL of autoclaved liquid Komada broth

(2,10). After 10 days, mycelium was vacuum-filtered through EMD® Miracloth™ and ground to powder in liquid nitrogen with a mortar and pestle. DNA extraction was performed on total mycelium with the Qiagen® DNeasy Plant Mini Kit ™ using the manufacturer's protocol. For the direct DNA extraction method, total DNA from 300 mg of soil was extracted with a Mo Bio® PowerSoil® DNA Isolation Kit (no. 12888-50, Carlsbad, 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, whereas 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 non-inoculated fresh DP744 root tissue and ground in a sample mesh containing 3 ml of GEB3 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 (Figure 3.4).

Multiple Samples with Agdia AmplifyRP® Acceler8™. A total of 300 mg of symptomatic root tissue of greenhouse-grown, FOVr4-inoculated DP744 plants was mixed in the following proportions with non-inoculated healthy root tissue (1:0, 1:1, 1:10, 1:25, 1:50, and 1:100). Samples were ground in a sample mesh bag containing 3 ml of GEB3 buffer and applied to the AmplifyRP® Acceler8™ kit. The same assay was conducted with FOV race 4-infected cotton plants (Phytogen 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.

3.4 DISCUSSION

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 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 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 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. This could be due to compounds in the soil that 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. The kit is more sensitive at detecting FOV race 4 DNA than conventional PCR. The kit detected DNA from FOV race 4 at concentrations as low as 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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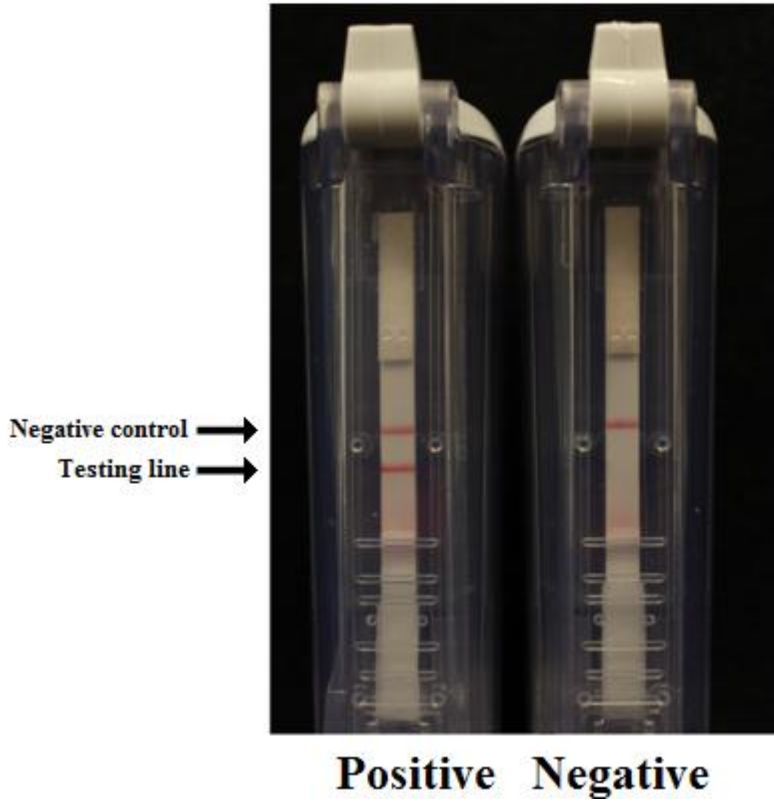

Figure 3.1. Detection of FOV race 4 in infected cotton with an AmplifyRP® Acceler8™ amplicon detection chamber.

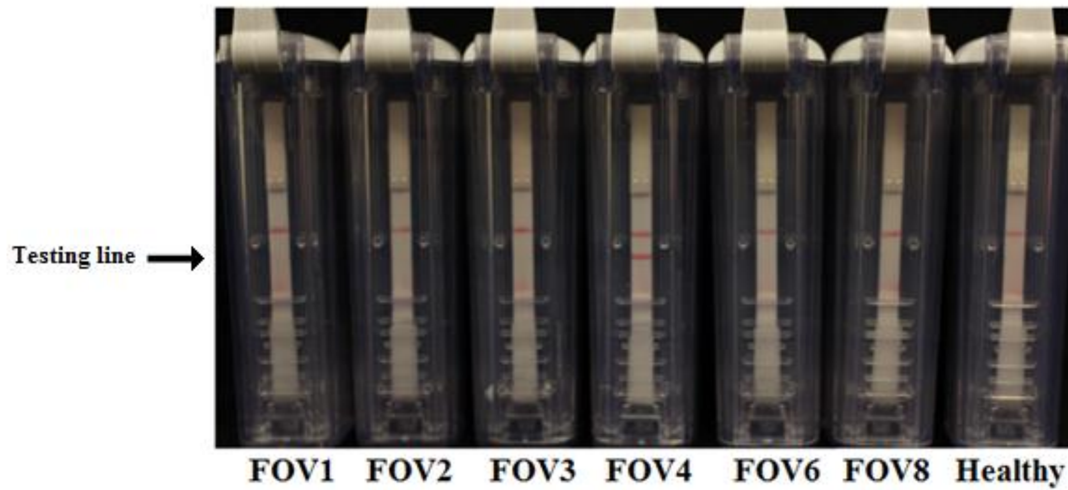

Figure 3.2. Detection of FOV race 4 in inoculated Deltapine 744 cotton tissue by an AmplifyRP® Acceler8™ Kit. FOV races 1, 2, 3, 6, and 8 resulted in no reaction. Plants mock-inoculated with water served as the healthy control.

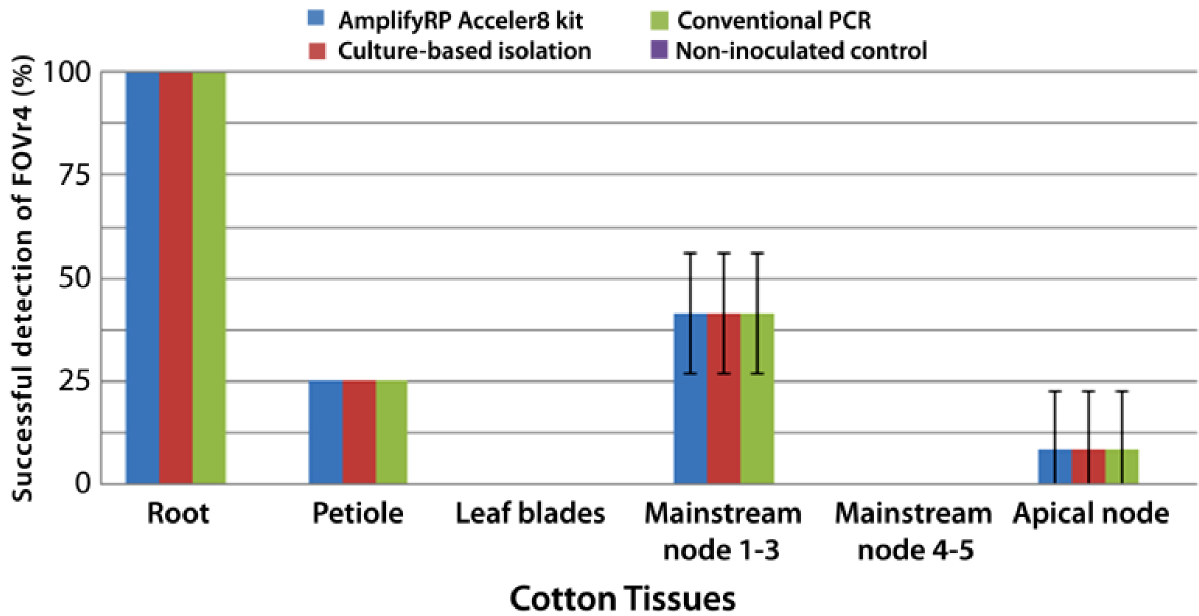

Figure 3.3. Detection of FOVr4 in various cotton tissues by AmplifyRP® Acceler8™ kit, conventional PCR, and culture-based isolation. Values represent the average number of successful detections of FOV race 4 out of four replications of three independent trials (12 samples). For detection by conventional PCR, we used published FOV race 4 specific primers (R4f: 5'GCTCCGTGTCWGAGCTTCTT and R4r: 5'GTTATGCTCCACGATGAG-CA). For detection by culture-based isolation, FOV race 4 was isolated on APDA, followed by DNA extraction and detection by FOV race 4 specific primers. Error bars are standard deviations.

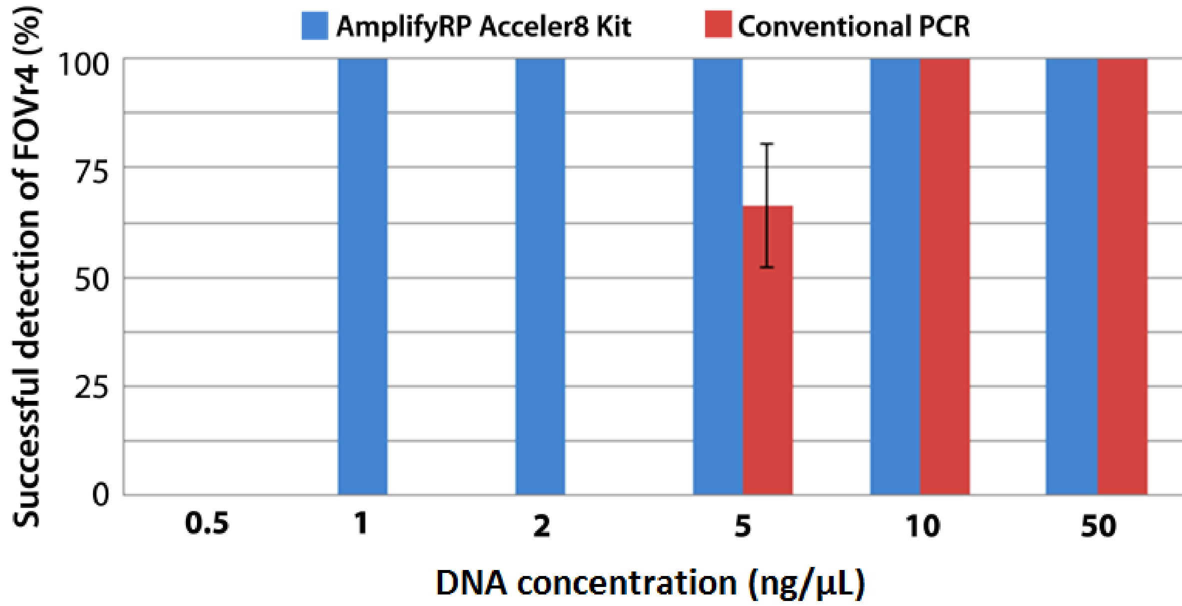

Figure 3.4. Sensitivity of AmplifyRP® Acceler8™ kit and conventional PCR to varying concentration of FOVr4 DNA. Values represent the average number of successful detections of FOV race 4 out of four replications of three independent trials (12 samples). For detection by conventional PCR, we used published FOV race 4 specific primers (R4f: 5'GCTCCGTGTCWGAGCTTCTT and R4r: 5'GTTATGCTCCACGATGAG-CA). Error bars are standard deviations.