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PLANT PATHOLOGY AND NEMATOLOGY

Seed Transmission of *Fusarium oxysporum* f. sp. *vasinfectum* Race 4 in California

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ABSTRACT

***Fusarium oxysporum* f. sp. *vasinfectum* (Atk.) W.C. Snyd. & H. N. Hans. race 4, a biotype highly virulent on certain Pima cotton (*Gossypium barbadense* L.) cultivars, was detected in California in 2001. The propensity of this disease to appear in isolated spots in previously uninfested fields has given rise to several hypotheses regarding potential mechanisms of disease dispersal. One of these hypotheses is that the disease may be spread through the planting of infected seed. In independent assays using two methodologies, the fungus was detected in acid-delinted Pima cotton seed from plants in known race 4-infested field sites. Seed was either plated directly onto Komada's agar or incubated en masse in a selective liquid medium. DNA isolated from the recovered fungi was amplified with race 4-specific primers. With both approaches, seed from susceptible Pima cultivars was infected with race 4, albeit at different levels. These results suggest that infected seed has the potential of spreading race 4 within and among cotton production regions.**

INTRODUCTION

Seedborne pathogens pose significant challenges to balancing interests of agricultural trade and disease management. The movement of seed for commerce and germplasm exchange greatly increases opportunities for long distance dispersal and permanent establishment of pathogens in new areas (Baker and Smith, 1966; McGee, 1995; Elmer, 2001). Confirming that a pathogen is seedborne and determining the incidence of seed infection in a production area are important steps toward assessing the risks of spreading the pathogen (Gabrielson, 1988).

Fusarium oxysporum Schltd.:Fr. f. sp. *vasinfectum* (Atk.) W.C. Snyd. & H. N. Hans., the cause of Fusarium wilt of cotton (*Gossypium* spp. L.), occurs in most major cotton production regions of the world. Seed transmission of this pathogen has been demonstrated in many parts of the world including the United States (Elliott, 1923; Taubenhaus and Ezekiel, 1932), India (Kulkarni, 1934), China (Chiu and Chang, 1982), Africa (Lagiere, 1952; Wickens, 1964), and Australia (Kochman et al., 2003). However, attempts to detect *F. oxysporum* f. sp. *vasinfectum* in seed from California, an important seed production region, were unsuccessful (Smith et al., 1981). In 2001, the discovery of *F. oxysporum* f. sp. *vasinfectum* race 4 in the San Joaquin Valley renewed interest in the possibility that infected seed could potentially spread this race to noninfested areas. Race 4 of *F. oxysporum* f. sp. *vasinfectum* has been identified as being highly virulent on most commercially available Pima (*G. barbadense* L.) cultivars (Kim et al., 2005) and can also damage Acala (*G. hirsutum* L.) cultivars (Ulloa et al., 2006). Unlike race 1, which is widespread in California (Kim et al., 2005) and the U.S. (Kappelman, 1983), race 4 does not require an interaction with the root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood, to cause significant damage to cotton. Consequently, losses to disease are not restricted to the sandy soils favorable for survival of *M. incognita* (Kim et al., 2005). The objective of this study was to assess the potential for acid-delinted cottonseed from fields in the San Joaquin Valley to carry viable propagules of the *F. oxysporum* f. sp. *vasinfectum* race 4. Preliminary data have been presented previously (Bennett et al., 2007).

MATERIALS AND METHODS

Cottonseed was collected in 2005 and 2006 from naturally infected plants grown in fields confirmed to be infested with race 4 (Hutmacher, unpublished).

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In 2005, seed was collected from three cultivars of Pima (Phytogen 800, Dow AgroSciences, Indianapolis, IN; DP 340 and DP 744 (Delta and Pine Land Company, Scott, MS) and one cultivar of Acala (Phytogen 72). The plants sampled in all fields exhibited foliar symptoms and vascular discoloration typical of Fusarium wilt. Seed of DP 340 and DP 744, and Phytogen 72, were collected in Fresno and Tulare Counties, respectively. Seed of Phytogen 800 were collected from both Fresno and Kern Counties. In 2006, a race 4-infested field of the susceptible experimental Pima cultivar 4126 (Bayer CropScience, Research Triangle Park, NC) in Kern County was overhead-irrigated in an attempt to create a humid environment favorable for seed infection. This cultivar has since been removed from the breeding program of Bayer CropScience. Water was applied by sprinklers for three 8-h periods during late flowering and boll formation at the end of September. Watering periods were separated by one or two days. Upon lint maturity, seed was harvested from the lower canopy (≤15 cm above ground level) from plants exhibiting wilt symptoms. In 2006, seed was also collected from a naturally infested field of DP 744 in Fresno County. After acid-delinting, seed was stored at -20° C (non-DP744 cultivars) or 5.5° C (DP 744) until assays were conducted four to eighteen months after harvest.

Fungal isolations for *F. oxysporum* f. sp. *vasinfectum* were made from individual seeds as well as a group of seeds (mass isolations). For isolations from individual seeds, seeds were surface-sterilized by one-minute treatments each in 95% ethanol followed by 10% solution of household bleach (0.6% NaOCl, w/v) and two rinses in sterile distilled water. A maximum of ten seeds per plate was placed on Komada's medium (Komada, 1975) and incubated at room temperature under a 12-h photoperiod maintained with fluorescent lights. To confirm the identification of isolates, single-spore cultures were obtained by plating spore dilutions onto ¼ -strength potato dextrose agar (¼-PDA), examining the plates under a dissecting microscope, and transferring individual germinated conidia to fresh plates of ¼-PDA. If colony morphologies indicated the possibility of more than one isolate of *Fusarium* originating from a seed, all variants were assayed. A total of 19,082 seeds were assayed from the 2005 season: 5730 seeds of Phytogen 800 from Fresno County, 4500 seeds of Phytogen 800 from Kern County, 5842 seeds of DP 340, and 3010 seeds of Phytogen 72. Seeds (6660) from the overhead-irrigated Pima plants in 2006 were also individually screened.

Mass isolations were performed in a using a *Fusarium*-selective liquid medium (D-glucose, 20.7 g; L-asparagine, 1.2 g; $K_2HPO_4 \cdot 3H_20$, 1.2 g; $MgSO_4 \cdot 7H_20$, 0.5 g; yeast extract, 0.5 g; NaCl, 0.1 g; distilled water to 1 L) (Kim et al., 2005). After the medium was autoclaved and cooled to 50° C, the following selective agents were added: ampicillin, 0.25 g; pentachloronitrobenzene (PCNB), 10 ml (5 mg/ml EtOH stock solution); and, streptomycin sulfate, 2 ml (0.15 g/ml stock solution). Aliquots of 50, surface-sterilized seeds each of acid-delinted DP 744 were placed in 250 ml flasks containing 120 ml of the selective medium. Flasks were gently shaken for 10-14 days at room temperature. A total of 400 seeds from each of the two DP 744 seed lots (collected in 2005 and 2006) was placed in eight flasks of 50 seed each. The procedure was repeated with 500 additional seed from the DP 744 seed lot collected in 2005. The mycelia in each flask were harvested by filtration through Miracloth (EMD Biosciences, San Diego, CA) and rinsed with sterile distilled water. Seed of the Acala cultivar Maxxa (Bayer CropScience, Research Triangle Park, NC) from a commercial field not known to harbor race 4 were used as a negative control in mass isolations. Flasks of Maxxa seed cultured with a 6-mm diameter plug from a race 4 colony cultured on PDA were used as positive controls.

Race 4 isolates of *F. oxysporum* f. sp. *vasinfectum* were identified using race 4-specific PCR primers (Yang et al., 2006). DNA was obtained from *Fusarium*-like colonies isolated from individual seed by using an XL2000 sonicator (Misonix, Inc., Farmingdale, NY). Approximately 25 mm^2 of mycelium was scraped from a colony with a sterile toothpick, suspended in 500 µl of 10 mM Tris-HCl (pH 8.0), and sonicated for 20 seconds at power level 5. Mycelia from race 4 colonies and known non-race 4 colonies (other fusaria or fungi) were used as positive and negative sonication controls, respectively. DNA from single-spore cultures and mass-seed mycelia were obtained using the FastDNA Kit and FastPrep Instrument (QBiogene, Irvine, CA) or the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.,Carlsbad, CA) according to the manufacturers' instructions. The PCR was conducted in final volumes of 10 µl, using 10-20 ng of template DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, and 0.25 units of GoTaq DNA polymerase (Promega,

Madison, WI). Positive (race 4 DNA) and negative (non-race 4 DNA) controls were included for each set of PCR. Thermocycler conditions were 10 cycles of 30 s each at 94°C, 59°C, and 72°C, followed by 25 cycles of 30 s at 90°C, 30 s at 50°C, and 15 s at 72°C. PCR products from samples and controls were separated on 1.5% TAE agarose gels and examined for the 208 bp product amplified in race 4 DNA (Yang et al., 2006).

RESULTS AND DISCUSSION

In the individual seed assays, few fusaria (6 of 19,082 seeds) were found in the seed lots from diseased plants in infested fields collected in 2005, and none were *F. oxysporum* f. sp. *vasinfectum* race 4. More fusaria (410 of 6,660 seeds) were found on Bayer 4126 Pima cottonseeds from the overhead-irrigated plants of the 2006 season, and one isolate tested positive with the race 4 primers. This isolate was confirmed as race 4 by screening multiple single-spore isolates with the PCR assay. With the mass isolations from Pima cultivar DP 744, the race 4-specific amplicon was obtained in four and five of the eight flasks with seed collected in 2005 and 2006, respectively. In the second assay of the 2005 seed lot, race 4 was detected in seven of ten flasks. The race 4-specific amplicon was never obtained from the negative control flasks of Maxxa seed. Amplicons were consistently detected from the positive controls of Maxxa seed co-cultured with a mycelial plug of race 4.

These results indicate that *F. oxysporum* f. sp. *vasinfectum* race 4 is able to infect cottonseed in California under field conditions. Race 4 was frequently detected in the Pima cultivar DP 744 using mass isolations, and infrequently found in other cultivars using individual seed isolation. While DP 744 is among the most susceptible cultivars to race 4 (Kim et al., 2005), it is unclear why it would be more frequently infected than other cultivars. Other investigators have observed higher incidences of seed infection in susceptible cultivars than in resistant cultivars (Hillocks, 1983; Kochman et al., 2003), but the mass isolation medium and technique may have enhanced growth of race 4 more than Komada's medium. Seed infection by *Fusarium* spp. declines over time when seed has been stored at room temperature (Elliott, 1923; Kochman et al., 2003), but all seed assayed in this experiment was stored at -20° C (non-DP 744 seed) or 5.5° C (DP 744). Both are temperatures that are commonly used to store *F. oxysporum* for many years, and assays were done within 18 months of seed harvest. Since the cultivars were not assayed sequentially, it was also observed that recovery of *Fusarium* spp. did not decrease over time (data not shown). Additional studies of the incidence of seed infection in DP 744 and other cultivars, will be conducted to determine the effect of isolation technique (mass and individual seed) on recovery of *F. oxysporum* f. sp. *vasinfectum.*

Reports of seed infection levels from symptomatic plants have ranged from undetectable in California (Smith et al., 1981) to a high of 47% in Tanzania (Perry, 1962). However, most reports of seed infection levels have been around 10% or less, particularly when seed from highly or moderately resistant cultivars or nonsymptomatic plants are assayed or included among seed from susceptible, symptomatic plants (Hillocks, 1992). In addition, low rates of infection may have contributed to conflicting conclusions about the ability of *F. oxysporum* f. sp. *vasinfectum* to survive acid-delinting and subsequently infect seedlings. Before the report of Elliott (1923), pathologists had been unable to demonstrate seed infection and transmission by any race (Fulton, 1907; Gilbert, 1921), and several attempts after Elliott were also unsuccessful (Fahmy, 1927; Neal, 1928). The issue appears to have been unresolved until Taubenhaus (1932) confirmed seed infection and transmission in Texas. Cottonseed infected with *F. oxysporum* f. sp. *vasinfectum* has since been reported from most major production areas worldwide (Kulkarni, 1934; Hillocks, 1992; Kochman et al., 2003).

The specific races of *F. oxysporum* f. sp. *vasinfectum* involved in most of these reports can only be inferred since pathogenicity and diagnostic tests to determine race were either not yet developed or were not conducted with seed-derived isolates. One exception is Kochman et al. (2003), who tested seed-derived fusaria with PCR primers to identify the two vegetative compatibility groups (Bentley et al., 2000) causing disease losses in Australia. Prior to its discovery in California, race 4 had only been reported from India (Armstrong and Armstrong, 1960), China (Skovgaard et al., 2001), and Uzbekistan (Fernandez et al., 1994). Because seed infection has been reported from both China (Chiu and Chang, 1982) and India (Kulkarni, 1934), albeit without identifying the isolates to race, seed infection by race 4 may have been demonstrated previously.

Baker and Smith (1966) identified several reasons why seed-transmission of pathogens is important, including: 1) infected seed greatly increases opportunity for long distance dispersal; 2) susceptible hosts will select for newly introduced isolates possessing greater virulence over preexisting strains; and 3) the distribution of infected seed within a seed lot creates the opportunity for multiple, randomly distributed foci of primary infections in fields and faster spread compared with a single introduction at the field margin. Once established into a new area, several soilborne fungal pathogens – including *F. oxysporum* f. sp. *vasinfectum* – appear capable of persisting indefinitely by living saprophytically or by colonizing nonhosts (Smith and Snyder, 1975; Smith et al., 2001). For these reasons, the potential consequences of distributing cottonseed originating from areas infested with race 4 need to be carefully considered until effective management tactics become available. These results suggest that dispersal of race 4 by infected seed is a possible explanation for the patterns of occurrence of this disease in newly infested fields. These results also illustrate the need for additional study of the mechanisms controlling *Fusarium* dispersal by infected seed.

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DISCLAIMER

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