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FUSARIUM WILT OF COTTON IN CALIFORNIA: CHARACTERIZATION AND PCR-BASED DETECTION OF RACE 4

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Abstract

The development of specific primers for the detection of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) race 4 is described. Primers were designed from the sequence of an AFLP marker unique to race 4 and screened against a collection of California FOV isolates, virulent Australian FOV isolates, and various fungi isolated from cotton tissue. The amplification of DNA by the primers was unique to FOV race 4. Characterization of disease development caused by FOV race 4 was tested at different inoculum densities, ranging from zero to 10^6 spores per gram of soil. Results for two Upland cotton cultivars, Phytogen 72 and Ultima EF, and three Pima cotton cultivars, Delta Pine 340, Delta Pine 744, and Phytogen 800, suggest an inoculum threshold between 10^3 and 10^4 spores per gram of soil for disease development.

Introduction

Fusarium wilt of cotton occurs in most cotton-growing areas of the world. Two recent developments have elevated the status of this disease, which was historically a minor issue, to national importance. One is the emergence of a virulent race of the fungus in Australia (Davis 1996) and the other is the recent discovery of race 4, a particularly virulent biotype, in the San Joaquin Valley (Kim 2005). The former threatens California cotton production because Australian cotton seed was imported into California as cattle feed; the latter is important because it will no doubt spread in soil on farm equipment, in addition to possible seed transport. The ability to detect these fungi in seed and soil is necessary to prevent movement of the pathogen into and within California. The development of FOV race 4-specific primers will enable the rapid detection of the pathogen by polymerase chain reaction (PCR).

FOV race 4 causes varying degrees of disease in different cotton cultivars and under different conditions. Pima cultivars generally show more susceptibility to race 4 than Upland cultivars, however, the Pima cultivar Phytogen 800 appears to be resistant to race 4 in the field. In order to characterize disease development as a factor of inoculum density, a greenhouse study was conducted on two Upland and three Pima cotton cultivars planted in infested soil ranging from zero to 10^6 spores per gram of soil.

Materials and Methods

Race 4 Primer Development

Representative isolates of the seven known world races of FOV were included in the study (Table 1). DNA was extracted from liquid cultures of each isolate using Qiagen's DNeasy plant mini kit. DNA was quantified by spectrophotometry and concentrations were then equalized. Amplified Fragment Length Polymorphism (AFLP) reagents including enzymes and primers were purchased from Invitrogen and reaction conditions were from protocols developed by Vos et al. (1995). Samples were run on polyacrylamide gel and visualized with Applied Biosystems's GeneScan software and by silver stain. Polymorphisms unique to race 4 were detected from the gel images (Figure 1). Candidate polymorphic bands were excised from the gel and cloned in order to separate individual fragments within the band. Cloned fragments were DNA sequenced and primers were developed from sequence analysis.

Table 1. Isolates included in AFLP screen for unique Race 4 markers.

Isolate	Race	Source

ATCC 16613	4	ATCC
FOV 14	4	Fresno Co.
FOV 56	4	Kern Co., Buttonwillow
FOV 59	4	so. Kern Co., east of 99
FOV 63	4	Fresno Co.
FOV 66	4	Fresno Co., Stanislaus/Mt Whitney
S-6	4	Tulare Co., Paige/99
100B	4	Fresno Co., Vanguard
ATCC 16421	1	ATCC
FOV 2	1	Tulare Co.
FOV 5	1	Kern Co.
FOV 8	1	Kings Co.
FOV 30	1	Tulare Co.
FOV 31	1	Tulare Co.
FOV 50	1	A. Bell
ATCC 16611	2	ATCC
ATCC 16612	3	ATCC
FOV 3	3	Tulare Co.
ATCC 36198	6	ATCC
ATCC 31665	8	ATCC
FOV 1	8	Tulare Co.
FOV 7	8	Kings Co.
FOV 32	8	Tulare Co.
FOV 16	A1	Cecil Plains, Queensland, Australia
FOV 19	A2	Boggabilla, New South Wales, Australia

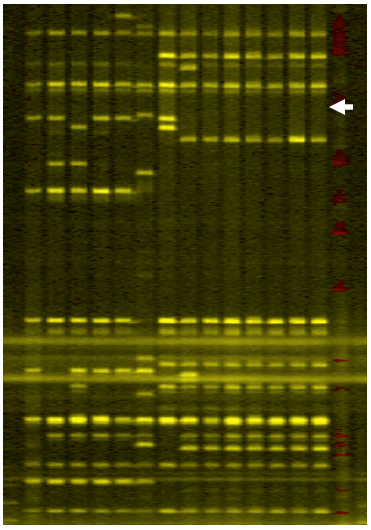

Figure 1. Polyacrylamide gel image of a candidate AFLP marker for race 4. Lanes 1 through 7 respectively: race 1, race 2, race 6, FOV 30 (race 1), FOV 31 (race 2), race 3, race 8; lanes 8 through 14: race 4 isolates from California; lane 15: DNA ladder. Arrow indicates a candidate marker for race 4 at approximately 380 bp.

Race 4-specific primers (R4f and R4r) were tested on DNA extracts from California race 4 isolates, Australian races A1 and A2 isolates, as well as the other known world races: 1, 2, 3, 6, and 8. The primers were also tested on DNA extracts of race 4-infected cotton tissue and soil, and the following fungi isolated from cotton tissue: *Alternaria sp.*, *Aspergillus sp.*, *Cladosporium sp.*, *Epicoccum sp.*, *Fusaium equiseti*, *Fusarium sp*., and *Trichothecium sp*.

Race 4 Disease Characterization

Two Upland (Phytogen 72 and Ultima EF) and three Pima cotton cultlivars (Delta Pine 340, Delta Pine 744, and Phytogen 800) were grown at inoculum densities ranging from zero to 10^6 spores per gram of soil. The treatments were arranged in a randomized complete block design consisting of four replicates of each cultivar at each inoculum density. The experiment was repeated once. Disease development was measured by scoring height, fresh weight, number of nodes, vascular discoloration, symptoms, and number of spores recovered per gram of stem tissue.

Results

The race-4 specific primers, R4f and R4r, developed from AFLP marker sequences successfully amplified an expected 208 bp product from DNA extracts of California race 4 isolates; DNA from representative isolates of all other races and other fungi tested did not amplify (Figure 2). In order to screen for the presence of PCR inhibitors in samples that were not amplified by primers R4f and R4r, a PCR of the samples was run using primers ITS4 and ITS5. All samples were amplified. The primers also amplified DNA from young, infected cotton tissue but failed to amplify DNA from older plants or from soil. The primer sequences were R4f 5'gctccgtgtcwgagcttctt and R4r 5'gttatgctccacgatgagca.

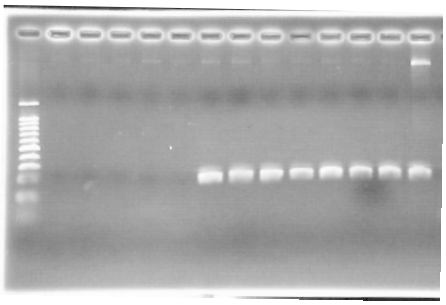

Figure 2a. PCR products of all FOV races using race 4-specific primers. Lane 1: 100 bp DNA ladder; lanes 2 through 6 respectively: race 1, race 2, race 6, race 3, race 8; lanes 7 through 14: race 4 isolates from California.

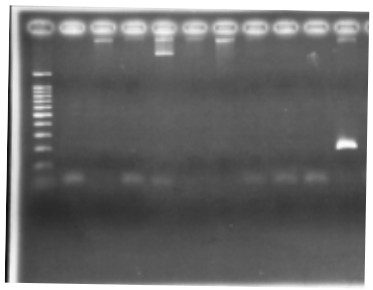

Figure 2b. PCR products of fungi isolated from cotton using race 4-specific primers. Lane 1: 100 bp DNA ladder; lanes 2 through 11 respectively: *Aspergillus*, *Fusarium equiseti*, *Alternaria*, *Trichothecium*, *Alternaria*, *Fusarium sp.*, *Alternaria*, *Epicoccum*, *Cladosporium*, FOV race 4.

Results of the greenhouse study suggest that a threshold for disease development occurs between 10^3 and 10^4 spores per gram of soil. At the 95% confidence level, significant interaction was detected between cultivar and inoculum density. For all parameters tested, disease was more severe at the increasing levels of inoculum. The most susceptible cultivar, Delta Pine 744, developed more severe disease and at lower inoculum densities than the other cultivars. The resistant Pima cultivar, Phytogen 800, showed the least disease development at all inoculum densities, however, mild symptoms were observed at 10^4 spores per gram of soil and greater.

Discussion

Using the race 4-specific primers, PCR amplification was optimal for DNA extracted from cultured isolates, but amplification of DNA from infected-cotton tissue was weak. Generally, younger cotton plants allowed stronger amplification of target DNA than older tissue, possibly due to greater amounts of PCR inhibitors in older

and woodier plant tissues. The primers have been tested on race 4-infected soil DNA extracts without success. An improved protocol for optimal DNA extraction and PCR conditions will likely improve amplification from older plant tissue and soil. One of the goals is to adapt the procedures for Real-Time PCR applications to quantify inoculum concentrations in field soil. Economic thresholds of inoculum may then be detected.

References

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