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CHARACTERIZATION OF CALIFORNIA ISOLATES OF *FUSARIUM OXYSPORUM* F. SP. *VASINFECTUM*

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Abstract

Because there is concern over the introduction of highly virulent Australian strains of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) into California, it is necessary to characterize local strains of the fungus to document the movement of genotypes in and out of important cotton-producing areas. Regulatory decisions may rely on such data regarding movement of plant material between states or countries where different strains exist. To accurately describe the strains that presently occur in California, various genetic methods were employed. Based on partial sequences of translation elongation factor and beta-tubulin genes and restriction enzyme digestion of the IGS nuclear r-DNA, Australian isolates of FOV do not exist in California. Californian isolates fell into four lineages. One group of several isolates consisted of the lineage of races 1 and 2, which are closely related to each other; a second group was represented by two isolates that may or may not belong to the race 3 lineage; a third group, race 4, was commonly collected in California; and a fourth group, race 8, was represented by two isolates. Race 4 was highly virulent on Pima in greenhouse pathogenicity tests and was considerably less virulent on Acala; isolates belonging to the lineage of races 1, 2, and 6 were relatively virulent on Acala cotton but not Pima. Race 8 and race 3(?) were weakly pathogenic on both Acala (Maxxa and Phy-72) and Pima (DP-744 and Pima S-7).

Introduction

Fusarium wilt of cotton occurs in a wide area of the San Joaquin Valley of California. Symptoms of the disease include wilting, yellowing, and necrosis of leaves. Internally, the xylem is dark brown. If infected early, plant death may result. The fungus survives in the soil long-term and once a field is infested, it will likely remain so since the fungus reproduces on the roots of a number of crops and weeds. Eradication from fields is probably impossible. Many races, determined by their virulence to a differential set of cotton lines and species (Smith et al., 1981), non-cotton hosts and, more recently, by genetic analyses, are currently recognized worldwide (Assigbetse et al., 1994 and Skovgaard et al., 2002). In California, most isolates of *Fusarium oxysporum* f. sp. *vasinfectum* (FOV) cause relatively mild symptoms on Upland cotton unless plants are infected with root knot nematodes. In fact, the degree of symptom expression by California FOV isolates in Upland cotton is related to feeding damage by the nematode. For this reason, control of root knot nematode (crop rotation, nematicides, etc.) is key to managing the disease. Some cotton varieties are tolerant to the root knot nematode and/or Fusarium wilt, which aids in disease management. However, in recent years Pima varieties have suffered significant losses in the absence of nematodes. This observation has lead to this investigation on the diversity and pathogenicity of California isolates of FOV on Upland and Pima varieties.

Because FOV is seed-borne, there is concern over the introduction of Australian strains of the fungus into California on shipments of cotton seed for cattle feed. Two strains possibly unique to Australia occur there that are more virulent than California isolates and require no interaction with the root knot nematode (Davis et al., 1996 and Kochman et al., 1996). The introduction of one of these fungi could cause havoc in the California cotton industry.

Recently, the California Cotton Ginners and Growers Association has asked the USDA and California Department of Food and Agriculture to halt the importation of cottonseed from Australia for feed and seed. Currently, cottonseed is imported into California in 25,000 ton shipments from Queensland, a Fusarium-infested cotton-growing area of Australia. The seed is unloaded onto the docks in California and into trucks that deliver to dairies throughout the Central Valley. Although the seed is fumigated in Australia before it is loaded onto ships, California cotton producers assert that fumigation is not 100% effective, especially given the fact that FOV may be internal in the seed.

In a recent ruling by the Pest Exclusion Branch, Plant Health and Pest Prevention Services, California Department of Food and Agriculture, import permits were allowed to continue despite arguments by the cotton industry. One of the reasons cited for the extension of the permits was the possibility that the Australian strains already exist in California. Hence, the need to characterize California and Australian isolates of FOV. Such data are needed to document the movement of genotypes in and out of California and support regulatory decisions regarding movement of plant material between areas where different strains exist. This project promises to fill this gap in our knowledge about California isolates of FOV.

Materials and Methods

Twenty-four California FOV isolates were collected from cotton fields in the San Joaquin Valley in the last two years or much earlier (some of the isolates in the culture collection of J. E. DeVay and S. N. Smith were collected 20+ years ago). Each California isolate used in this study, to the best of our knowledge, originated in a different field. In addition to the California collection, a USDA permit was obtained to import three isolates of each of the two strains that exist in Australia and four strains representing races 1-4 from ATCC. Using Qiagen kits, DNA was extracted from liquid cultures of all 34 isolates. Several methods were employed to characterize the isolates: 1) the IGS region of nuclear ribosomal DNA was amplified with conserved primers and digested with the restriction enzymes RsaI and ScrF (S. Bentley, *personal communication*); 2) amplification and sequencing of regions of the elongation factor (EF-1, 5': ATG GGT AAG GAA GAC AAG AC and EF-2, 3': GGA AGT ACC AGT GAT CAT GTT (O'Donnell et al. 1998)); and 3) amplification and sequencing of regions of the beta-tubulin gene (BT5, 5': GCT CTA GAC TGC TTT CTG GCA GAC C and BT-3, 3': CGT CTA GAG GTA CCC ATA CCG GCA). In addition to molecular characterization, all California isolates were screened for pathogenicity on cotton in the greenhouse (by agreement with APHIS, we cannot inoculate any plants with the Australian isolates, but they have already been screened in that country, in any case). All isolates were stored on dried cellulose disks.

Sequences were aligned manually in MacClade 3.0 (Maddison and Maddison, 1992). Maximum parsimony heuristic searches were performed in PAUP* 4.b10 (Swofford, 1998) with the following general settings: all characters with equal weight, starting trees via stepwise addition, random taxa addition sequence with 1,000 replications, MAXTREES set to autoincrease, TBR branch swapping on, MULTREES on, and gaps treated as "missing". The trees were rooted with a saprophytic isolate of *Fusarium oxysporum* (isolate Fo-1502) from soil in California frequently planted to vegetables (T. G. Gordon, Univ. of California, Davis). Congruence between combined data sets was evaluated using the subprogram Partition-Homogeneity test in PAUP.

In greenhouse pathogenicity tests, cotton seedlings with a single true leaf were root dip-inoculated in a conidial suspension of 1 X 10^5 spores/ml for 10 minutes. Controls were dipped in water. Plants were then transplanted into pasteurized sand:peat moss (1:1) in individual 15 cm-diameter pots. Greenhouse temperatures were maintained at 22-27 C. After approximately 5 weeks, plants were removed from the pots and cut at the soil line. The vasculature near the soil line and distal several centimeters was examined for discoloration typical of Fusarium wilt. Roots were washed and the dry weights of both the plant tops and root system were determined.

Results

All three genetic methods successfully differentiated groups of California isolates from one another and the Australian isolates from all California strains. The digestion of the IGS region of the nuclear rDNA demonstrated that the Australian strains are unique and that California strains can be divided into several groups (data not presented since these groups were near identical to those listed below). The Partition-Homogeneity test indicated no significant difference between trees generated by the EF gene and the beta-tubulin gene; therefore, the analysis was performed on the combined sequences. One of two most parsimonious trees is presented in Fig. 1. Of 1097 characters, 20 were parsimony informative (the EF sequences accounted for most of the polymorphisms). Five lineages are apparent. Lineage I is represented by 14 California isolates and the race 4 isolate (16613) from ATCC. Lineage II consists of six California isolates and races 1 (16421) and 2 (16611) from ATCC. The third lineage, which is represented by two California isolates, is identical to race 8 in other data bases (data not presented). The fourth lineage of two California isolates may or may not be related to race 3 (16612) from ATCC. It is possible that these two isolates from California represent an undescribed race. The fifth lineage consists of the Australian strains.

In pathogenicity tests conducted in the greenhouse, differences in virulence to cotton were apparent among the various races. Certain California isolates, e.g., 9 and 14 (race 4 isolates), were highly virulent on Pima but not Acala whereas other isolates, e.g., 4, 8, and 10 (race 1,2 lineage), were relatively virulent on Acala and less so on Pima (Table 2 and 3). Representative isolates of race 8 and race 3(?) were weakly pathogenic on both Acala (Maxxa and Phy-72) and Pima (DP-744 and Pima S-7) cotton plants.

Discussion

Based on our collection to date, several distinct lineages and races of *Fusarium oxysporum* f. sp. *vasinfectum* occur in California. Race 4, which is thought to have Asian origins (Skovgaard et al., 2002), was commonly recovered from three counties in the San Joaquin Valley (Table 4). Isolates of the lineage of races 1 and 2 were recovered on six occasions from three counties. These races may represent a near identical group which originated in the Americas (they differ on their ability to cause disease on non-cotton hosts but cause identical responses on various cotton varieties and species). Variability in pathogenicity tests with differentials and non-cotton hosts has been problematic with these strains; some researchers propose that these races be lumped into a single race (Assigbetse et al., 1994). Race 8, which may have its origin in Asia, was cultured from cotton in two counties in California. The fourth group, which was tentatively assigned to a lineage with race 3 from the Nile

Valley, was recovered from Tulare Co. More data are needed for the proper race assignment of these isolates. They could represent an as-yet-undefined race. The Australian strains, which represent a unique lineage of *F. oxysporum* f. sp. *vasinfectum*, were not recovered in our California survey. Based on our work to date, there is no evidence that Australian strains occur in California, which could be used to support regulatory issues that prevent its movement.

This work has immediate utility for the cotton industry in California. The identification of strains in a field can be used to make decisions on the type of cotton to plant. For example, if a field is infested with race 4, the grower can probably safely grow an Acala cotton variety, especially if nematodes are controlled. Pima varieties, however, should be avoided in fields infested with race 4. In contrast, Pima is a good choice is the grower is faced with an infestation of races 1 or 2. In that situation, good nematode control may result in plants that are so lightly affected that the disease goes unnoticed. Based on the results of greenhouse pathogenicity tests in this study, the other races that occur in California pose little or no threat to the industry. It is clear, however, that virulent strains which exist elsewhere in the world are potential threats to the California cotton industry. This seedborne pathogen has obviously spread from one corner of the globe to the other and will probably continue to do so.

References

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Table 1. Isolates of *Fusarium oxysporum* f. sp. *vasinfectum* used in this study.

Taxon number	Source	Reference	Origin (from cotton)
1	Smith/DeVay	2LS	Tulare Co., CA
2	Smith/DeVay	0HS	Tulare Co., CA
3	Smith/DeVay	5LS	Tulare Co., CA
4	Smith/DeVay	7HS	Tulare Co., CA
5	Smith/DeVay	2F 2'c-1	Kern Co., CA
6	Smith/DeVay	3F 3'a-1	Kern Co., CA
7	Smith/DeVay	2F1'a-5	Kings Co., CA
8	Smith/DeVay	3F3'e-1	Kings Co., CA
9	this study	59	Kings Co., CA
10	this study	88-2	Kern Co., CA
11	this study	91-1	Tulare Co., CA
12	this study	92-2	Fresno Co., CA
13	this study	93-1	Fresno Co., CA
14	this study	94-1	Fresno Co., CA
15	this study	95-1	Fresno Co., CA
16	S. Bentley	24230	Queensland, Australia
17	S. Bentley	24500	NSW, Australia
18	S. Bentley	24595	Queensland, Australia
19	S. Bentley	24391	NSW, Australia
20	S. Bentley	24597	NSW, Australia
21	S. Bentley	24598	NSW, Australia
S6	this study	F6	Tulare Co., CA
S7	this study	F7	Tulare Co., CA
S8	this study	F8	Fresno Co., CA
S9	this study	F9	Fresno Co., CA
S12	this study	F12	Fresno Co., CA
S14	this study	F14	Tulare Co., CA
100b	this study	100b	Kings Co., CA
103a	this study	103a	Kings Co., CA
104d	this study	104d	Kings Co., CA
16421	ATCC	16421 (race 1)	USA
16611	ATCC	16611 (race 2)	USA
16612	ATCC	16612 (race 3)	Egypt
16613	ATCC	16613 (race 4)	India

Table 2. Pathogenicity tests of *Fusarium oxysporum* f. sp. *vasinfectum* isolates on Acala and Pima cotton. Values are based on a disease index scale where 0 = no vascular discoloration to 4 = intense browning of vascular tissue (little or no growth occurred in plants with a rating of four).

	Acala		Pima	
Isolate	Maxxa	Phy-72	DP-744	Pima S-7
None	0.2 b	0 c	0.2 c	0 c
3	1.0 ab	0.8 ab	0.7 bc	1.0 b
7	1.3 ab	1.3 ab	1.2 b	0.8 b
10	1.6 ab	2.5 a	1.2 b	1.0 b
14	2.2 a	1.0 ab	4.0 a	4.0 a

Values are the means of 6 reps in each of two duplicate trials; means in each column followed by the same letters are not significantly (P = 0.05) different according to least significant difference test.

Table 3. Pathogenicity tests of *Fusarium oxysporum* f. sp. *vasinfectum* isolates on Acala and Pima cotton. Values are based on a disease index scale where 0 = no vascular discoloration to 4 = intense browning of vascular tissue (little or no growth occurred in plants with a rating of four).

	Acala		Pima	
Isolate	**Maxxa**	**Phy-72**	**DP-744**	**Pima S-7**
None	0	0	0	0
4	3.5 a	2.5 a	1.2 b	0
8	1.8 b	3.5 a	0.2 b	0
9	0	1.8 ab	4.0 a	3.6 a
11	0	0	0	0

Values are the means of 6 reps in each of two duplicate trials; means in each column followed by the same letters are not significantly (P = 0.05) different according to least significant difference test.

Table 4. The occurrence of *Fusarium oxysporum* f. sp. *vasinfectum* in California counties. Number of isolates, each recovered from an individual field, is in parentheses.

Races 1, 2, & 6* (Americas)**	**Race 3 (?) (Nile Valley)**	**Race 4 (Asia)**	**Race 8 (Asia)**
Kern Co. (3)	Tulare Co. (2)	Fresno Co. (7)	Kings Co. (1)
Kings Co. (1)		Kings Co. (3)	Tulare Co. (1)
Tulare Co. (2)		Tulare Co. (3)	

* Races 1, 2, and 6 are indistinguishable based on the methods used in this study. Others have proposed that these might represent a single group (Assigbetse et al., 1994).
**Possible origin of races.

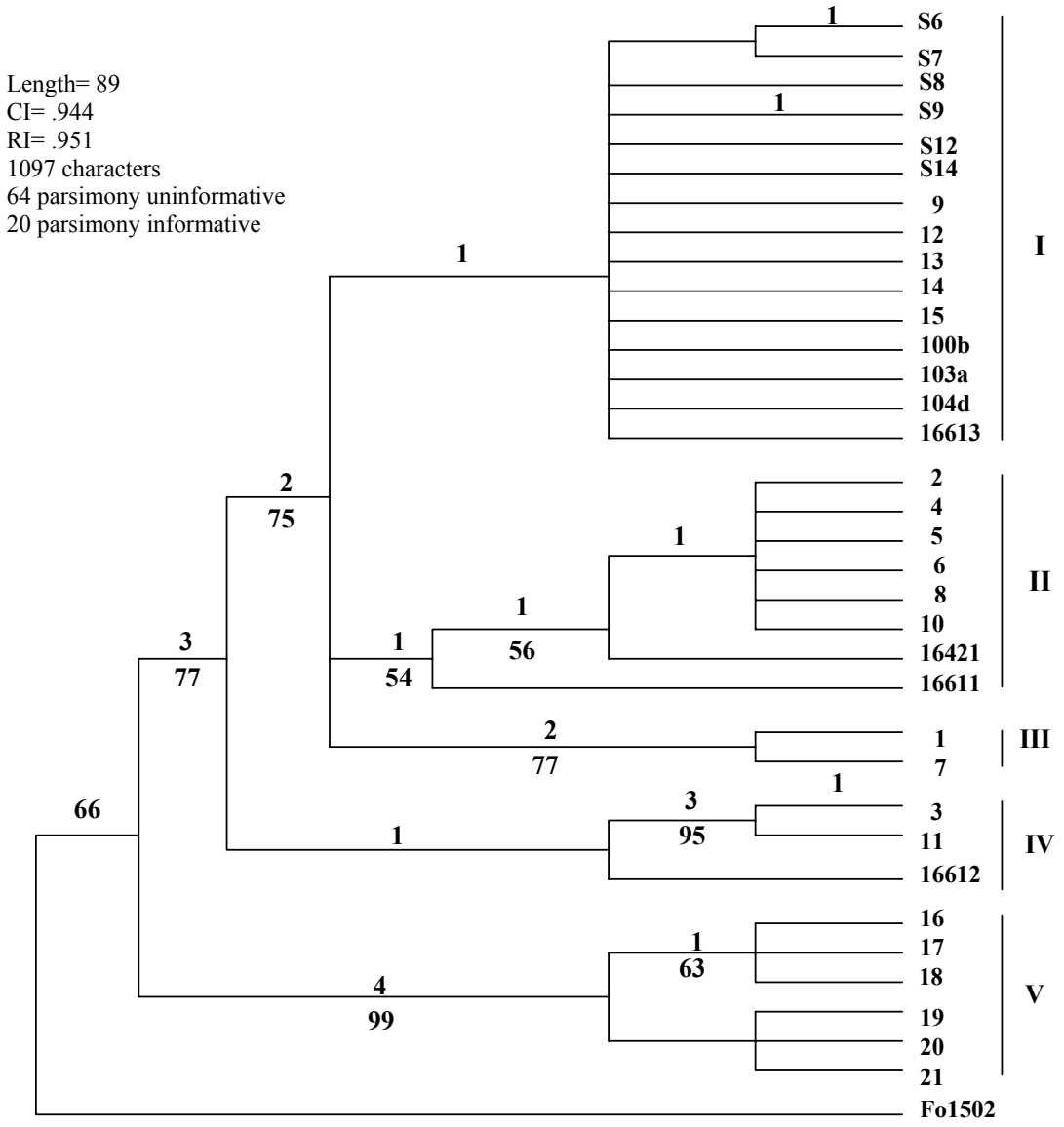

Figure 1. One of two most parsimonious trees inferred from combined analysis of partial sequences of translation elongation factor and beta tubulin genes. Branch lengths are shown above the branches and bootstrap frequencies of > 50% from 1,500 replications are shown below each branch. The tree is rooted with a saprophytic *Fusarium oxysporum* (Fo-1502) from California soils. Five lineages (I to V) of *Fusarium oxysporum* f. sp. *vasinfectum* races are identified. The relationship of isolates 3 to 11 to 16612 (race 3) is not well supported and should be considered tentative.