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PATHOGENIC COMPARISONS OF *FUSARIUM* ISOLATES FROM DISEASED HAWAIIAN *ACACIA KOA*

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ABSTRACT

Thirty-two isolates of *Fusarium* from Hawaiian *Acacia koa* exhibiting wilt/dieback disease symptoms were compared for their pathogenic potential on young Douglas-fir germinants in controlled laboratory tests. Only three isolates (one each of *F. oxysporum*, *F. solani*, and *F. subglutinans*) exhibited low virulence. All others, including isolates of *F. semitectum* and *F. equiseti*, were non-pathogenic. All isolates were capable of colonizing succulent germinant tissues. Varying levels of root decay and post-emergence damping-off were caused by some test isolates. Many infected germinants survived without disease symptoms throughout the 14-day test. *Fusarium* isolates from *Acacia koa*, although capable of colonizing non-host plant tissues, may be specifically adapted to their koa hosts.

INTRODUCTION

Recent investigations indicated that several different *Fusarium* species were routinely isolated from Hawaiian *Acacia koa* Gray exhibiting wilt and dieback symptoms (James 2004a). Based on previous research (Anderson et al. 2002; Gardner 1980), *Fusarium oxysporum* Schlecht. f.sp. *koae* is considered the putative causal organism of the disease. Isolates of *F. oxysporum* were recently obtained from some sampled diseased plants, and were especially common within roots and adjacent soil (James 2004a). This species was not found on or within seeds and seedpods from diseased trees. Several other *Fusarium* spp., including *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas, *F. semi-tectum* Berk. & Rav., *F. solani* (Mart.) Appel. & Wollenw., *F. sambucinum* Fuckel, *F. avenaceum* (Fr.)

Sacc., *F. sporotrichioides* Sherb., and *F. equiseti* (Corda) Sacc. were isolated at varying levels from diseased plant tissues, adjacent soil, and seed/seedpods. With the exception of *F. solani* (Daehler and Dudley 2002), none of these other *Fusarium* spp. were previously reported on either diseased or healthy koa. Therefore, their role, if any, in eliciting disease on koa or any other plant host was unknown.

During early 2005, tests were initiated to evaluate potential of several of these *Fusarium* species to elicit disease on inoculated koa seedlings at the Mauna-wili research site on Oahu. These tests will be completed by late spring and their results should help clarify the role of several *Fusarium* spp. in the koa wilt/dieback disease syndrome.

A laboratory procedure was developed several years ago to quickly evaluate potential of *Fusarium* spp. to elicit plant diseases (James 1996). In this procedure, young germinants of Douglas-fir (*Pseudotsuga menziesii* var. *glauca* [Beissn] Franco) are exposed to selected *Fusarium* isolates for a maximum of 14 days. Aggressiveness of isolates are compared on the basis of germinant survival, presence of disease and a numerical virulence rating (James 1996). Aggressiveness of selected *Fus-arium* isolates from Hawaiian koa was compared using this procedure.

MATERIALS AND METHODS

Tested *Fusarium* isolates were obtained from koa seeds/seedpods, diseased seedlings and trees, and soil adjacent to roots of declining trees (table 1). A representative cross section of isolates obtained from different parts of diseased *Acacia koa* were included in the test; 32 *Fusarium* isolates were tested (table 2). Fifteen of these isolates were *F. oxysporum*, the putative cause of koa wilt/decline (Anderson et al. 2002; Gardner 1980). Five *F. solani*, three *F. subglutinans*, and two each of *F. semitectum* and *F. equiseti* were also tested.

The laboratory pathogenicity technique used (James 1996) was based on exposing young Douglas-fir germinants to test fungal isolates and recording production of disease symptoms. Cornmeal-perlite inoculum was used for all tests because it had proved successful in several previous investigations of *Fusarium* pathogenicity (James 2000, 2004b; James and Perez 1999, 2000; James et al. 1997, 2000). This inoculum was prepared using the procedures of Miles and Wilcoxin (1984). Perlite, an inert, inorganic, siliceous rock of volcanic origin commonly used in potting mixtures, was the matrix for fungal growth. 150 g of yellow cornmeal was moistened with 300 ml warm 1% potato dextrose agar (PDA), to which 75 g of perlite were added. The mixture was placed into glass vials to about 2/3 capacity which were then autoclaved for 60 min. at 121^{o}C. After cooling, vials were inoculated with about 10 ml spore suspension of each test fungus (produced by adding sterile, distilled water to 14-day-old cultures grown on PDA). Vial caps were left loose to allow aeration. Vials were incubated in the dark for at least 21 days, after which the fungus had thoroughly colonized the perlite /corn-meal mixture. After incubation, in-oculum was removed from vials and dried in open petri plates within a cabinet. Inoculum dried within 5-7 days and did not become contaminated with

other microorganisms because the food base was completely colonized by the inoculated fungus. Once dry, inoculum was stored in sterile, plastic vials and refrigerated until needed.

Each test involved exposing 24 germinants to specific fungal isolates within 23-ml vials. Each vial was filled to about 2/3 capacity (2.5 g) with dried, pre-mixed coconut-vermiculite (coir) media (Grace/Sierra Horticultural Products, Milpitas, CA). The media was not autoclaved. One high germination Douglas-fir seedlot (lot 4458 - USDA Forest Service Nursery, Coeur d'Alene, Idaho) was used for all tests. Seeds were soaked in a 2-part bleach and 3-part water solution for 10 min. (Wenny and Dumroese 1987), rinsed 48 hrs. in running tap water, and stratified 21 days at 2-3^{o}C. After stratification, seeds were placed on filter paper moistened with sterile water in petri plates. Seeds were incubated under 12-hr. diurnal fluorescent light cycles at about 24^{o}C and monitored daily for germination; they were considered germinated when their radicle was at least 3 mm long.

Fungal inoculum (colonized perlite /cornmeal) was ground to fine powder with mortar and pestle. Exactly 0.05 g of the powder was added to each vial containing dried media. This resulted in an approximately 1:50 w/w mixture of inoculum to media. Controls consisted of exposing 24 germinants to non-inoculated perlite within vials; they were evaluated like germinants inoculated with fungal isolates.

Vials containing inoculated germinants were incubated at about 24^{o}C on a lab bench; fluorescent light was provided for about 8-10 hours daily. Tests ran a maximum of 14 days. Three days after inoculation, germinants were first checked for disease symptoms. During this inspection, germinant roots were reoriented downward into the medium, if necessary. Germinants were then checked for disease symptoms daily until the end of the test. Standard post-emergence damping-off (figure 1) and root decay occurring below the ground line without visible mycelial production (figure 2) were the two types of disease symptoms. After 14 days, surviving germinants (without noticeable disease symptoms) were examined to determine if their roots had grown to the bottom of the inoculation vial; their roots were also examined for decay and/or necrotic lesions. Roots from all inoculated germinants were washed, surface sterilized in 10% bleach (0.525% aqueous sodium hypochlorite) and incubated on a selective agar medium for *Fusarium* (Komada 1975) to determine if they were colonized by the inoculated isolate.

A numerical test score was assigned to each inoculated germinant based on duration of survival (without disease symptoms) within inoculated vials, occurrence and type of disease, reisolation of inoculated fungal isolate, and primary root growth within the vial (James 1996). The maximum score possible (germinant killed within 3 days by the test isolate) was 100; the minimum (germinant not infected within 14 days) was zero. The average rating for all germinants tested for a particular isolate was used to compare isolates. Virulence ratings were assigned based on average test scores: non-pathogenic = <40; low virulence = 41-60; moderate virulence = 61-80; high virulence = >80.

Table 1. Isolation characteristics of *Fusarium* isolates from diseased Hawaiian *Acacia koa* compared for pathogenic aggressiveness.

Fusarium Isolate Number	Isolation Location
0421	Seeds and Seedpods
0422	Seeds and Seedpods
0424	Diseased Seedlings; Stem & Roots
0425	Diseased Seedlings; Stem & Roots
0429	Rhizosphere Soil on Roots of Diseased Trees
0430	Soil Adjacent to Roots of Diseased Trees
0431	Stems and Cankers of Diseased Trees
0432	Branches of Diseased Trees
0433	Fine Roots and Root Interiors of Diseased Trees

RESULTS

Most tested *Fusarium* isolates from diseased *Acacia koa* were non-pathogenic on young Douglas-fir germinants (table 2). One isolate each of *F. oxysporum*, *F. solani*, and *F. subglutinans* was considered weakly virulent in these tests; all others were non-pathogenic. However, germinant tissues were usually colonized by test isolates, i.e., test fungi were reisolated from inoculated germinants. In addition, some tested isolates caused disease symptoms during the 2 week test. The most common symptom was root decay (figure 2) with no noticeable effect on the above-ground portion of the germinant other than reduced growth. Average virulence scores for the five tested *Fusarium* spp. were fairly similar, with those of *F. subglutinans* (isolated from koa seeds and seedpods) somewhat higher than the other species. In several cases, there were fairly large differences in virulence scores among isolates within a particular species. For example, virulence scores of isolates of *F. oxysporum* ranged from 18 to nearly 50. When considered as a group, however, the tested *Fusarium* isolates were non-pathogenic.

DISCUSSION

Fusarium oxysporum is the putative cause of dieback and wilt of *Acacia koa* seedlings and trees on several Hawaiian islands (Anderson et al. 2002; Gardner 1980). Pathogenicity of isolates obtained from diseased plants on healthy koa seedlings was previously confirmed (Anderson et al. 2002; Gardner 1980) and very little genetic variation was found when populations of *F. oxysporum* from koa were compared (Anderson et al. 2004). More recent investigations indicated that several other *Fusarium* spp., in addition to *F. oxysporum*, were commonly isolated from diseased koa plants, seeds and seedpods, and nearby soil (James 2004a). Twelve isolates representing four of these other species (*F. solani*, *F. subglutinans, F. semitectum*, and *F. equiseti*) and 15 isolates of *F. oxysporum* were tested for aggressiveness on young Douglas-fir germinants in a laboratory assay.

Figure 1. Post-emergence damping-off of young Douglas-fir germinant inoculated with *Fusarium oxysporum* from *Acacia koa*. Germinant was exposed to the test fungus for 6 days.

Figure 2. Douglas-fir germinant root decayed by *Fusarium oxysporum* obtained from *Acacia koa* after exposure to the fungus for 7 days.

Table 2. Virulence comparisons on young Douglas-fir germinants among *Fusarium* isolates from diseased Hawaiian *Acacia koa*.

Species[1]	Isolate No.	Average Days Alive[2]	Percent Diseased[3]	Virulence Score[4]	Virulence Rating[5]
FOXY	0422K	13.7	17.4	22.0	Non-pathogen
FOXY	0424D	13.8	12.5	21.9	Non-pathogen
FOXY	0424G	12.8	65.2	38.0	Non-pathogen
FOXY	0424K	12.9	43.5	33.0	Non-pathogen
FOXY	0424M	12.9	50.0	35.4	Non-pathogen
FOXY	0425C	14.0	8.7	18.0	Non-pathogen
FOXY	0425H	13.2	69.6	38.0	Non-pathogen
FOXY	0430G	12.5	62.5	36.9	Non-pathogen
FOXY	0430H	13.2	75.0	35.8	Non-pathogen
FOXY	0430K	13.1	30.4	28.3	Non-pathogen
FOXY	0431H	11.7	81.8	49.1	Low Virulence
FOXY	0432F	13.8	29.2	22.9	Non-pathogen
FOXY	0433E	12.7	47.6	34.3	Non-pathogen
FOXY	0433K	13.1	45.0	32.5	Non-pathogen
FOXY	0433M	13.5	13.0	20.9	Non-pathogen
AVE FOXY		**13.1**	**43.3**	**31.0**	**Non-pathogen**
FSOL	0421Q	13.7	4.2	19.4	Non-pathogen
FSOL	0424F	12.4	43.5	34.1	Non-pathogen
FSOL	0429P	12.8	33.3	31.2	Non-pathogen
FSOL	0429Q	10.4	59.1	51.1	Low Virulence
FSOL	0433I	13.1	31.8	29.8	Non-pathogen
AVE FSOL		**12.5**	**33.9**	**32.9**	**Non-pathogen**
FSUB	0421Z	9.2	79.2	59.6	Low Virulence
FSUB	0421AA	14.0	4.3	18.0	Non-pathogen
FSUB	0421CC	12.8	33.3	30.9	Non-pathogen
AVE FSUB		**11.9**	**40.0**	**36.7**	**Non-pathogen**
FSEM	0421D	13.4	30.0	22.8	Non-pathogen
FSEM	0432I	13.0	30.4	28.0	Non-pathogen
AVE FSEM		**13.2**	**30.2**	**25.6**	**Non-pathogen**
FEQU	0421J	12.4	39.1	33.9	Non-pathogen
FEQU	0424N	13.4	8.7	18.5	Non-pathogen
AVE FEQU		**12.9**	**23.9**	**26.2**	**Non-pathogen**
AVE ALL *FUSARIUM*		**12.9**	**38.8**	**31.3**	**Non-pathogen**
CONTROL		**14.0**	**0.0**	**8.8**	**Non-pathogen**

[1]FOXY = *Fusarium oxysporum*; FSOL = *F. solani*; *F. subglutinans*; FSEM = *F. semitectum*; FEQU = *F. equiseti*

[2]Maximum of 14 days; minimum of 3 days

[3]Percent of Douglas-fir germinants with damping off or root decay

[4]Range 0-100

[5]Virulence ratings: < 40 = Non-pathogenic; 41-60 = Low virulence; 61-80 = Moderately virulent; > 80 = Highly virulent

Similar tests using *Fusarium* isolates obtained from diseased or healthy conifer seedlings, forest nursery soil, and styrofoam containers used to grow several crops of container seedlings indicated that there is often a wide range of virulence within particular *Fusarium* spp. (James and Perez 1999, 2000; James et al. 2000). The test procedure used provides ideal conditions for evaluated isolates to infect, colonize, and induce disease symptoms on young germinants. Therefore, if isolates are incapable of causing much disease or require many days to elicit host responses, it is likely that those isolates would not be capable of causing disease under more natural conditions either within nurseries or on field sites.

Using criteria developed for the laboratory test procedure only three of the thirty-two tested *Fusarium* isolates from diseased Hawaiian koa plants exhibited virulence on young Douglas-fir germinants. These isolates, one each of *F oxysporum*, *F. subglutinans* and *F. solani*, were only weakly virulent under test conditions. All other tested isolates were considered non-pathogenic.

It is likely that the *Fusarium* isolates obtained from Hawaiian koa plants are specialized to that host. It is likely that not all of these isolates are pathogens of their koa hosts. Some may be secondary colonizers of diseased or insect-damaged tissues (Daehler and Dudley 2002); others may exist as endophytes within host tissues without adversely affecting colonized plants (Hoff et al. 2004; Stone et al. 2000). Those capable of inciting disease symptoms within colonized hosts are probably specifically adapted to koa and incapable of causing diseases of other plant species. For isolates classified morphologically as *F. oxysporum* (Nelson et al. 1983), wilt-inducing pathogenic strains are probably capable of causing disease only on koa plants. Host-specific pathogenic isolates of *F. oxysporum* are usually placed within a sub-species taxonomic designation called *formae speciales* [f.sp. = species form](Armstrong and Armstrong 1976; Gordon and Martyn 1997). The putative pathogen causing koa wilt is classified as f.sp. *koae*, on the basis of its ability to cause disease specifically on koa plants (Anderson et al. 2002, 2004; Gardner 1980). It is unknown if any of the *F. oxysporum* isolates tested on Douglas-fir germinants are actually pathogenic on koa, and therefore classified as f.sp. *koae*. Some tested isolates are undoubtedly saprophytes, which often comprise large proportions of *F. oxysporum* populations (Bao and Lazarovits 2001; Bao et al. 2002; Gordon and Martyn 1997). Others may have pathogenic potential on koa. However, none of the tested isolates had high potential to incite disease on Douglas-fir.

All tested *Fusarium* isolates were capable of infecting and colonizing young, succulent Douglas-fir tissues. In many cases, no disease resulted from this colonization. When disease did occur, the most common symptom was decay of the radicle tip after it had grown into inoculated media. The above-ground portion of such affected germinants lacked any indication of disease. In a very few cases, post-emergence damping-off was evident. Inoculated isolates were usually reisolated from test germinants. Therefore, it appears that all tested

Fusarium species were capable of rapidly colonizing non-host plants, but often did not cause disease. This may be due to genetic characteristics of the tested isolates (Baayen et al. 2000; Lori et al. 2004; Pasquali et al. 2003; Stewart et al. 2004), i.e, they lack the necessary pathogenicity genes for inciting disease on Douglas-fir. It may also be due to responses of non-host tissues to fungal invasion (Di Pietro et al. 2003; Roncero et al. 2003). In any event, it seems likely that *Fusarium* isolates pathogenic on Hawaiian koa plants are specifically adapted to that host and may not be able to induce disease on other non-related plant species.

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