United States Department of Agriculture | Forest Service | Northern Region | 200 East Broadway P.O. Box 7669 Missoula, MT 59807

Forest Health Protection

Numbered Report 07-08

November 2007

COMPARATIVE VIRULENCE OF HAWAIIAN *FUSARIUM OXYSPORUM* ISOLATES ON *ACACIA KOA* SEEDLINGS

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ABSTRACT

Sixty-one fungal isolates within the *Fusarium oxysporum* species complex isolated from *Acacia koa* plants or rhizosphere soil in Hawaii were tested for pathogenicity on young koa seedlings. Only about 15% of these were considered highly- or moderately-virulent in greenhouse inoculation tests. On the other hand, more than 50% of the tested isolates either exhibited low virulence or were considered non-pathogenic. Highly-virulent isolates were easily identified because they killed nearly all inoculated seedlings during the 90-day tests. Non-pathogenic isolates often did not cause disease symptoms on any of the 24 seedlings exposed per isolate. We have confidence that our testing procedure can be used to easily and consistently identify highly-virulent and non-pathogenic isolates within Hawaiian populations of *F. oxysporum* and may be used to screen koa families for potential disease reistance.

INTRODUCTION

Fusarium oxysporum Schlecht. is a taxon containing a complex of morphologically-similar *Fusarium* species that cause important plant diseases worldwide. Strains may vary widely genetically, although they usually cannot be differentiated morphologically (Gordon and Martyn 1997; Kistler 1997). Pathogenic strains of *F. oxysporum* are often quite host-specific and those capable of eliciting disease on particular host species are designated by a sub-specific taxon called a formae specialis (Armstrong and Armstrong 1975; Nelson and others 1981). However, saprophytic strains incapable of causing diseases are widespread and often occupy the same environmental niches as pathogenic strains (Gordon and Martyn 1997; Nelson and others 1981). Saprophytic strains cannot be differentiated from pathogenic strains unless either tested on susceptible hosts or

separated on the basis of specific genetic markers associated with virulence genes (Alves-Santos and others 2002; Bao and others 2002; Chiocchetti and others 1999; Gordon and Okamoto 1992). Pathogenicity testing is expensive and time-consuming, whereas genetic differentiation can usually be done more rapidly and can be less costly if genetic markers for specific pathosystems have been identified.

We have recently been investigating an important wilt/dieback disease of *Acacia koa* A. Gray in Hawaii. Koa is the most economically-important tree in many Hawaiian forest ecosystems, where it grows in moist habitats at elevations from 90 to 2100 m. High tree mortality following koa plantings due to this disease has restricted koa establishment, particularly in certain areas (Daehler and Dudley 2002). Koa wilt/dieback was first described in Hawaii by Gardner (1980). He completed Koch's postulates and determined that the primary cause of the disease was pathogenic strains of *F. oxysporum* which he designated f.sp. *koae*. Information on several aspects of disease impact and biology was subsequently investigated (Anderson and others 2002). An initial genetic analysis of several pathogenic Hawaiian isolates of *F. oxysporum* from diseased koa plants indicated potentially low genetic diversity, which may indicate recent introduction(s) of the pathogen into Hawaii (Anderson and others 2004). Subsequent genetic characterization of a much larger population of *F. oxysporum* from different sized hosts and islands indicated that the overall Hawaiian population within this species complex is likely quite diverse; at least six genetically-distinct clades were identified within a population representing more than 100 isolates from throughout Hawaii (Yang and others 2007).

We have routinely isolated *F. oxysporum* from roots and occasionally from stems and branches of large diseased koa trees (James and others 2007a), either diseased or healthy-appearing seedlings (James 2004; James and others 2007b), rhizosphere soil and occasionally from seeds and seedpods (James 2004; James and others 2006). Most of these isolates appear very similar morphologically, based on presence and structure of particular spores (microconidia, macroconidia, chlamydospores) and conidiophores (short, unbranched monophialides) (Nelson and others 1983). Most isolates also appear similar when grown in culture (aerial hyphae, sporodochia, pigment formation). Although preliminary characterization of *F. oxysporum* populations associated with Hawaiian koa has been done (Anderson and others 2002; Yang and others 2007), specific genetic markers associated with pathogenic strains have not yet been identified. We initiated greenhouse tests to determine pathogenic potential of selected *F. oxysporum* isolates obtained from Hawaiian koa in an effort to identify specific isolates that might be used to screen koa families for potential disease resistance and to quantify the prevalence of potential virulence within fungal populations.

MATERIALS AND METHODS

Sixty-one isolates identified as *F. oxysporum* based on morphological characteristics were selected for testing in greenhouse seedling inoculation experiments (Table 2 - Appendix). Three tests were conducted sequentially; two in 2006 and one in 2007.

For each selected isolate, fungal 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 perlite-cornmeal-PDA mixture was autoclaved at 121^{o}C for 60 min, cooled, inoculated with spore suspensions of test fungi, and incubated at about 24^{o}C in the dark for at least 15 days. After incubation, inoculum was 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 inoculated fungal isolates. Once dry, inoculum was refrigerated until needed.

Inoculum was ground to a fine powder and thoroughly mixed with commercial peat moss/perlite growing media (Sunshine Mix 4, Aggregate Plus, Sungro Horticulture, Belleview, WA) at a concentration of 1:50 (w/w). Inoculum-growing media mixtures were placed into plastic containers ("dibble tubes" – 115 mm^3) which were either new or had previously been sterilized by immersion in hot water (71^oC for 5 min).

Seeds of *Acacia koa* from one family were nicked at their distal end with nail clippers to break dormancy, soaked in water for about 12 hrs and sown into flats containing a 50:50 (v/v) mixture of vermiculite (Sta-Green Horticultural Vermiculite, St. Louis, MO) and perlite (Redco II, North Hollywood, CA), periodically watered, and monitored for germination. Following germination, when radicals were approximately they same length as cotyledons, they were carefully extracted from the flat and transplanted into the plastic containers with inoculum-growing media mixtures. Following transplanting, seedlings were watered to activate inoculum. For each tested isolate, four replications of six seedlings each were evaluated. Two fully replicated sets of 24 seedlings each were included as controls. These contained seedlings transplanted into peat/perlite growing media without fungal inoculum.

Transplanted seedlings were monitored for development of wilt and/or foliar chlorosis and necrosis. When seedlings were considered dead, they were carefully extracted from plastic containers, their roots thoroughly washed to remove adhering particles of growing media, and analyzed in the laboratory for root colonization by inoculated isolates. For this analysis, ten randomly-selected root pieces, each ap-proximately 5 mm in length, from each seedling were surface sterilized in a 10% bleach solution (0.525% aqueous sodium hypochlorite; 1 part standard household bleach in 10 parts water), rinsed in sterile, distilled water, and placed on a selective agar medium for *Fusarium* spp. (Komada 1975). Plates were incubated under diurnal cycles of cool, fluorescent light at about 24^oC for 7-10 days. Emerging fungi were compared with inoculated isolates to determine whether they were the same morphological species.

Tests were run for a maximum of 90 days. Heights of seedlings surviving to the end of the test were measured and their biomass (oven-dry weight - 100^oC for a minimum of 24 hrs - of roots and tops) determined. A few root pieces from surviving seedlings were incubated on the selective agar medium to confirm reisolation of the inoculated isolates.

RESULTS

Virulence ratings for individual isolates were assigned primarily on the basis of disease production, but also included average survival (number of days seedlings lived during the 90-day test) and average height of non-diseased seedlings. Categories of virulence ratings and the proportion of isolates falling within each category are summarized in table 1. A listing of individual isolates in the approximate order of the virulence based on the above criteria is compiled in table 3 (Appendix).

Only about 15% of the tested isolates exhibited high- or moderately-high virulence on young koa seedlings under our greenhouse inoculation conditions. More than half of the isolates exhibited low virulence or were considered non-pathogenic. The other tested isolates were somewhere in between. As expected, isolates classified as highly-virulent caused extensive disease and seedlings survived for shorter time periods. Differences in average seedling height among the six virulence categories were inconsistent. Our results indicated that the majority of the tested *F. oxysporum* isolates were not highly-virulent strains and therefore probably not responsible for the wilt/dieback disease of koa (table 1).

Table 1. Virulence of Hawaiian *Fusarium oxysporum* isolates on *Acacia koa.*

Virulence Rating	Number of Isolates	Percent of Isolates	Average Disease[1]	Average Survival[2]	Average Height[3]
High	5	8.2	89.2	53.2	10.8
Moderately-High	4	6.6	65.6	62.0	12.3
Moderate	15	24.6	50.5	70.5	13.0
Moderately-Low	4	6.6	34.4	81.4	12.9
Low	6	9.8	20.9	85.0	11.3
Non-pathogenic	27	44.2	4.2	88.8	12.8
Controls	2 Sets	-	4.2	87.7	12.6

[1]Percent of inoculated seedlings that became diseased
[2]Average number of days seedlings survived; maximum = 90 days
[3]Average height (cm) of surviving seedlings

DISCUSSION

The protocols we used to test virulence of *F. oxysporum* isolates on young *Acacia koa* seedlings were developed initially for use on conifer seedlings (James 1996; James and others 1989). Results on conifers were consistently reliable in locating virulent *Fusarium* isolates and separating them from non-pathogens (James and others 1989, 1997, 2000). Therefore, these procedures were adapted for use on koa seedlings with the ultimate goal of developing a reliable inoculation system that could routinely be used to screen koa families for resistance to highly-virulent *F. oxysporum* isolates.

Classification of isolates into virulence ratings of "high", "moderately high", etc. were fairly arbitrary. However, we believe that we were able to reliably identify those isolates at the extremes of our established virulence categories: highly-virulent and non-pathogenic. Highly-virulent strains identified by our inoculation protocols can be used to screen koa families for potential disease resistance.

We found that only a small percent of tested isolates could be classified as highly- or moderately-virulent. These isolates were readily identifiable because they killed the majority of inoculated seedlings. Most seedling mortality usually began about one month after inoculation and extended for the next 3-4 weeks. We suspect that it takes about a month for virulent isolates to infect roots, grow into vascular tissues, spread systemically throughout seedlings, and begin to initiate wilt symptoms by occluding water transport within vascular cells of inoculated seedlings (Beckman and others 1989, 1991; Nelson and others 1981). After this period of mortality, surviving seedlings often did not develop disease symptoms for the remaining test period.

We found that all tested isolates, even those considered non-pathogenic, always infected roots of inoculated seedlings. Inoculated roots exhibited no noticeable necrosis or discoloration, i.e., they were white and appeared completely healthy. However, they were extensively colonized by inoculated isolates, even to the point where no other fungi were detected during root assays. We suspect that non-pathogenic isolates were unable to successfully colonize

vascular systems and thus spread systemically throughout inoculated seedlings (Beckman and others 1989; Nelson and others 1981). Such isolates may have been restricted to root cortical cells where they existed as endophytes which did not adversely affect seedling health (Bloomberg 1966; Dhingra and others 2000).

Our results indicated that most *F. oxysporum* isolates obtained from koa trees displaying wilt or dieback symptoms were not highly specialized pathogens of koa. Isolates within the *F. oxysporum* species complex that are specifically capable of causing koa diseases are classified as *F. oxysporum* f.sp. *koae* (Gardner 1980). Highly-specialized pathogenic strains have usually evolved from non-pathogenic *F. oxysporum* populations (Gordon and Martyn 1997; Kistler 1997). Such isolates are so specialized that they are usually incapable of causing disease on other plant hosts, even those closely-related to their primary hosts (Buxton 1958; Ficcadenti and others 2002; Roncero and others 2003). In some cases, specific races of *F. oxysporum* within particular formae speciales evolve to the point that they can only elicit disease on distinct host cultivars (Awuah and others 1986; Baayen and others 1988; Ben-Yephet and others 1997).

Pathogenic and non-pathogenic *F. oxysporum* isolates can often be differentiated using particular molecular genetic markers (Bao and Lazarovits 2001; Bao and others 2002; Di Pietro and others 2003; Lori and others 2004). These can be used to quickly locate highly-virulent isolates within particular fungal populations associated with host plants (Bao and others 2002; Di Pietro and others 2003) and within soil (Edel and others 2001). Genetic analyses using amplified fragment-length polymorphisms (AFLPs) were completed on a population of *F. oxysporum* isolated from diseased koa plants, healthy seedlings, and rhizosphere soil (Yang and others 2007). Four highly-virulent isolates were included in this analysis; they clustered together in groups of two within two distinct clades. Pathogenicity testing of other genetically-characterized isolates is current underway. We hope to relate virulence on seedlings to genetic differentiation with the goal of identifying specific molecular markers that can be used to easily identify highly-virulent isolates within natural koa-associated *F. oxysporum* populations.

The high percentage on non-pathogenic *F. oxysporum* isolates identified in our tests may indicate high potential for biological control of highly-virulent strains. Non-pathogenic strains of this fungus have successfully been used as biocontrol agents in several cropping systems (Alabouvette and others 1993; Benhamou and Garand 2001; Blok and others 1997; Damicone and Manning 1982; Duijff and others 1999; Fravel and Larkin 2002; Fravel and others 2003). These strains successfully compete with and occupy niches normally colonized by pathogenic strains (Alabouvette and others 1993; Bao and Lazarovits 2001; Fravel and others 2003). They may often be more specifically adapted to control pathogenic strains of *F. oxysporum* than other, commercially-developed microorganisms (De Cal and Melgarejo 2001; Dumroese and others 1998; El-Hassan and Gowen 2006; Mousseaux and others 1998). Biological control may particularly be attractive in nurseries where non-pathogenic strains can be easily introduced into seedling production systems (James and Dumorese 2006; Stewart and others 2004). Such an approach holds promise in Hawaiian nurseries producing koa seedlings because we have found very high levels of infection by *F. oxysporum* on seedling stock (James 2004; James and others 2007b).

This work confirmed our expectation of the wide variability of fungal strains within the *F. oxysporum* species complex in Hawaii. Although most of these organisms appear morphologically similar, there are extensive genetic differences (Yang and others 2007) that affect pathogenicity on koa plants. We plan to expand our screenings to include many more *F. oxysporum* isolates to confirm proportions of populations capable of eliciting wilt or dieback disease of Hawaiian koa. We also want to have as many highly-virulent isolates as possible for screening koa families for potential disease resistance. This will improve

our confidence that selected resistant families will perform well after planting.

ACKNOWLEDGEMENTS

We acknowledge the USDA Forest Service, Forest Health Protection, Special Technology Development grant R5-2005-02 for financial assistance for this research.

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APPENDIX

Table 2. Number and source information for Hawaiian *Fusarium oxysporum* isolates tested for virulence on *Acacia koa* seedlings.

Isolate Number	Isolation Location
0424F	Maui; diseased seedling; fine roots
0429D	Maui; diseased seedling; rhizosphere soil
0429K	Maui; diseased seedling; rhizosphere soil
0430B	Maui; diseased seedling; rhizosphere soil
0431B	Maui; diseased seedling; stem
0433A	Maui; diseased seedling; fine roots
0433E	Maui; diseased seedling; fine roots
0433F	Maui; diseased sapling; secondary roots
0433K	Maui; diseased seedling; fine roots
0433M	Maui: diseased seedling; fine roots
0433N	Maui; diseased seedling; fine roots
0503I	Big Island; diseased tree; fine roots
0503J	Big Island; diseased tree; fine roots
0503L	Big Island; diseased tree; fine roots
0503M	Big Island: diseased tree; fine roots
0504B	Big Island; diseased tree; secondary roots
0505A	Big Island: diseased tree; fine roots
0528A	Oahu; diseased tree; fine roots
0528D	Oahu; diseased tree; secondary roots
0528F	Oahu; diseased seedling; fine roots
0531A	Big Island; diseased tree; fine roots
0531B	Big Island; diseased tree; secondary roots
0531F	Big Island; diseased tree; secondary roots
0531M	Big Island; diseased tree; fine roots
0531O	Big Island; diseased tree; tertiary roots
0531W	Big Island; diseased tree; secondary roots
0532A	Big Island; diseased tree; fine roots
0532P	Big Island; diseased tree; fine roots
0532V	Big Island; diseased tree; secondary roots
0533C	Big Island; diseased tree; fine roots
0533F	Big Island; diseased tree; secondary roots
0540A	Big Island; diseased tree; secondary roots
0540H	Big Island; diseased tree; secondary roots
0540K	Big Island; diseased tree; secondary roots
0540R	Big Island; diseased tree; secondary roots
0542F	Big Island; diseased tree; fine roots
0542H	Big Island; diseased tree; secondary roots
0542J	Big Island; diseased tree; secondary roots
0542O	Big Island; diseased tree; secondary roots
0543H	Big Island; diseased tree; secondary roots
0544G	Big Island; diseased tree; secondary roots
0544J	Big Island; diseased tree; fine roots
0544M	Big Island; diseased tree; secondary roots
0545A	Big Island; diseased tree; fine roots
0545G	Big Island; diseased tree; secondary roots

0545K	Big Island; diseased tree; fine roots
0554A	Big Island; diseased tree; secondary roots
0554H	Big Island; diseased tree; rhizosphere soil
0554L	Big Island; diseased tree; fine roots
0554N	Big Island, diseased tree; secondary]roots
0608A	Big Island; diseased tree; fine roots
0608G	Big Island; diseased tree; fine roots
0618A	Big Island; slightly diseased seedling; fine roots [nursery]
0619A	Big Island; slightly diseased seedling; fine roots [nursery]
0620A	Big Island; slightly diseased seedling; fine roots [nursery]
0621A	Big Island; slightly diseased seedling; fine roots [nursery]
0622A	Maui; diseased tree; fine roots
0623J	Big Island; slightly diseased seedling; fine roots [nursery]
0624A	Big Island; healthy seedling; fine roots [nursery]
0625J	Big Island; healthy seedling; fine roots [nursery]
0629A	Big Island; slightly diseased seedling; fine roots [nursery]]

Table 3. Virulence of Hawaiian isolates of *Fusarium oxysporum* on *Acacia koa* seedlings.

Isolate Number	Virulence Rating	Percent Disease[1]	Average Survival[2]	Average Height[3]	Average ODW[4]	Average Vigor[5]
0505A	High	95.8	49.7	8.0	0.170	1.00
0544J	High	91.7	46.8	10.0	0.130	2.00
0503J	High	91.7	56.5	12.0	0.130	1.00
0503I	High	87.5	56.4	11.3	0.143	0.67
0433M	High	79.2	56.4	12.6	0.252	0.60
0540K	Moderately High	70.8	51.7	12.9	0.289	1.00
0533C	Moderately High	66.7	70.3	14.7	0.373	0.71
0532A	Moderately High	62.5	62.3	11.2	0.283	0.89
0433A	Moderately High	62.5	63.8	10.2	0.161	1.00
0503M	Moderate	58.3	65.9	12.2	0.264	0.50
0431B	Moderate	58.3	66.2	12.4	0.219	0.50
0532P	Moderate	58.3	76.7	16.1	0.388	1.00
0528A	Moderate	54.2	61.5	12.0	0.221	0.82
0528D	Moderate	54.2	63.6	12.6	0.234	0.73
0429K	Moderate	50.0	68.6	10.5	0.172	1.00
0433K	Moderate	50.0	68.8	12.5	0.232	0.83
0433E	Moderate	50.0	69.5	14.8	0.323	0.50
0533F	Moderate	50.0	72.6	13.9	0.261	0.75
0540H	Moderate	50.0	77.9	12.6	0.277	1.00
0503L	Moderate	45.8	69.5	12.1	0.241	0.54
0532V	Moderate	45.8	72.8	12.6	0.217	0.85
0540R	Moderate	45.8	73.7	14.8	0.294	0.67
0540A	Moderate	45.8	80.2	15.5	0.352	0.71
0504B	Moderate	41.7	69.6	10.5	0.195	1.07
0531B	Moderately Low	37.5	81.8	13.9	0.253	1.07
0424F	Moderately Low	33.3	76.2	11.5	0.199	0.94
0531A	Moderately Low	33.3	83.3	11.8	0.260	1.06
0531F	Moderately Low	33.3	84.0	14.5	0.275	0.88

0531M	Low	25.0	85.9	14.5	0.274	0.74
0531W	Low	25.0	86.0	13.1	0.255	0.53
0531O	Low	20.8	83.0	14.9	0.285	0.75
0554A	Low	20.8	84.6	9.6	0.105	1.58
0542O	Low	17.4	84.2	8.2	0.093	2.00
0544M	Low	16.7	86.2	7.8	0.095	2.00
0545K	Nonpathogenic	8.3	84.3	7.7	0.095	2.09
0625J	Nonpathogenic	8.3	86.4	17.8	0.631	1.23
0433F	Nonpathogenic	8.3	87.0	7.5	0.098	1.95
0433N	Nonpathogenic	8.3	88.5	8.3	0.100	3.50
0430B	Nonpathogenic	8.3	89.3	8.7	0.098	2.09
0554H	Nonpathogenic	8.3	89.5	7.6	0.095	2.00
0623J	Nonpathogenic	4.2	87.3	20.2	0.824	1.13
0629A	Nonpathogenic	4.2	87.7	14.5	0.452	1.17
0608G	Nonpathogenic	4.2	87.7	18.1	0.540	1.26
0624A	Nonpathogenic	4.2	87.7	19.1	0.782	1.13
0608A	Nonpathogenic	4.2	88.0	17.8	0.604	1.13
0542H	Nonpathogenic	4.2	88.4	7.6	0.098	2.08
0545G	Nonpathogenic	4.2	88.6	9.1	0.100	1.96
0545A	Nonpathogenic	4.2	88.8	7.6	0.091	2.09
0542J	Nonpathogenic	4.2	89.3	8.5	0.096	2.00
0528F	Nonpathogenic	4.2	89.3	8.8	0.098	2.00
0543H	Nonpathogenic	4.2	89.4	9.1	0.093	2.09
0544G	Nonpathogenic	4.2	89.6	8.9	0.100	1.91
0429D	Nonpathogenic	4.2	89.8	8.3	0.093	1.65
0542F	Nonpathogenic	4.2	89.8	9.3	0.100	2.00
0554N	Nonpathogenic	4.2	90.0	6.5	0.098	2.09
0554L	Nonpathogenic	0	90.0	8.2	0.098	1.96
0618A	Nonpathogenic	0	90.0	19.4	0.646	1.00
0619A	Nonpathogenic	0	90.0	20.5	0.824	1.00
0620A	Nonpathogenic	0	90.0	21.0	0.855	1.04
0621A	Nonpathogenic	0	90.0	23.4	1.010	1.13
0622A	Nonpathogenic	0	90.0	23.5	1.060	1.04
Control 1	-	4.2	87.8	10.9	0.099	0.61
Control 2	-	4.2	87.6	14.3	0.385	0.91

[1]Percent of 24 inoculated seedlings per isolate displaying foliar wilt, chlorosis or necrotic symptoms.
[2]Average number of days [maximum = 90 days] inoculated seedlings survived without displaying disease symptoms.
[3]cm
[4]oven-dry weight (g) of both tops and roots
[5]average vigor rating based on a 5-point numerical system [0-4]

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