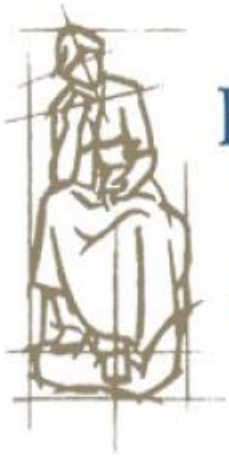

INVESTIGATING KOA WILT AND DIEBACK IN HAWAI'I

Pathogenicity of *Fusarium* species on *Acacia koa* seedlings

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ABSTRACT

Fusarium isolates obtained from diseased *Acacia koa* Gray (Fabaceae) plants, adjacent soil, and seeds and seedpods may or may not be pathogenic on young seedlings under greenhouse conditions. This includes isolates of *Fusarium oxysporum*, the putative cause of koa wilt and dieback disease ("koa wilt") in Hawai'i. We tested 10 *Fusarium* isolates, made up of 4 different species (*F. solani, F. subglutinans, F. oxysporum, F. semitectum*), for their pathogenic potential on koa seedlings under greenhouse conditions. All tested *Fusarium* isolates completely colonized seedling root systems and became systemic, spreading to aboveground plant tissues (stems, branches, and leaves). Virulence was quantified on the basis of disease symptoms (mortality, wilting, foliar chlorosis, or necrosis) and effects on seedling height, stem diameter, and root volume. Results varied, ranging from nonpathogenic to high levels of virulence. Pathogenic screening of many more isolates will be necessary to identify pathogens that can be effectively used to screen families of koa for potential resistance to the koa wilt and dieback disease that is seriously affecting this important Hawaiian tree species.

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KEY WORDS

reforestation, dieback disease, Fabaceae, *Fusarium oxysporum, Fusarium semitectum, Fusarium solani, Fusarium sterilihyphosum, Fusarium subglutinans*

NOMENCLATURE

Fungi: Nelson and others (1983)
Insects: ITIS (2007)
Plants: USDA NRCS (2007)

Native koa (*Acacia koa* Gray [Fabaceae]) is a dominant canopy tree in many Hawaiian forest ecosystems. Koa is present on the main Hawaiian Islands, growing in moist habitats at elevations from 90 to 2100 m (295 to 6890 ft). Koa has always been a vital part of Hawaiian culture; koa wood was used for building many original Hawaiian structures, as well as being prized for seafaring canoes. This important tree species is currently playing a primary role in an expanding Hawaiian wood industry, being used for producing furniture, musical instruments, bowls, surfboards, and handicrafts.

The major factor limiting establishment and maintenance of koa is a wilt disease that causes dieback that adversely affects tree survival and growth. Significant tree mortality following koa plantings due to this disease has restricted koa expansion, particularly in certain areas where disease severity is high. Koa wilt and dieback disease ("koa wilt") is putatively caused by the fungus *Fusarium oxysporum* f.sp. *koae*, which was first described in Hawai'i by Gardner (1980). Disease impacts and biology were subsequently investigated (Anderson and others 2002). Limited genetic analysis of several Hawaiian isolates of *F. oxysporum* f.sp. *koae* indicated potentially low genetic diversity, possibly due to recent introductions of pathogenic strains of this fungus into the state (Anderson and others 2004). If pathogenic strains were recently introduced from a source nonnative to Hawai'i, the severe disease impacts currently occurring may be a reflection of an invasive, well-adapted pathogen. Improved techniques for detection and management of this disease are urgently needed to limit pathogen spread and to reduce disease consequences. Several other *Fusarium* species, particularly *F. solani*, are routinely isolated from diseased koa seedlings and trees (Daehler and Dudley 2002; James 2004). In addition, 13 different *Fusarium* spp. were recently isolated from koa seeds and seedpods (James and others 2006). Some of these organisms may be involved in disease etiology and epidemiology and require testing for their ability to induce disease.

Family variation in koa field trials strongly suggests the presence of resistance to *F. oxysporum* f. sp. *koae* (Dudley 2002). Although koa wilt has significantly affected koa plantings on certain sites, some seed sources appear mostly unaffected. Therefore, genetic disease resistance likely occurs and may be widespread within some families. Frequency of resistance in natural koa populations is unknown but is expected to be low. Inoculation methods for screening potential disease resistance in seedlings are available (James and others 1989; James 1996; Anderson and others 2002). Rapid screening of many koa families would provide valuable information on resistance potential. Following laboratory and greenhouse experiments, outplanting field validation of resistance screening results will be needed (Sniezko 2003).

The present work was designed to evaluate pathogenic potential of selected *Fusarium* isolates associated with diseased koa plants and nearby soil to help clarify their potential roles in disease etiology. Tests were conducted under controlled conditions on koa seedlings within a greenhouse.

MATERIALS AND METHODS

Many isolates of *Fusarium* were obtained during routine isolations from koa seedlings, large trees exhibiting koa wilt symptoms, and seeds and seedpods. Isolates comprised several different *Fusarium* species (James 2004; James and others 2006), which were determined on the basis of morphological characterization (Nelson and others 1983). Some of these species have not previously been described on koa and their potential role in disease etiology is unknown. Therefore, seedling inoculation tests were initiated to help elucidate disease potential of selected representative isolates. Ten isolates encompassing 4 different *Fusarium* species were evaluated for their pathogenic potential (Table 1).

Fungal inoculum was prepared using the procedures of Miles and Wilcoxin (1984). Perlite, an inert, inorganic, siliceous rock of volcanic origin that is commonly used in potting mixtures, was the matrix for fungal growth. Yellow cornmeal (150 g [5.3 oz]) was moistened with 300 ml (10 fl oz) warm 1% potato dextrose agar (PDA), to which 75 g (2.6 oz) of perlite were added. The perlite-cornmeal-PDA matrix was autoclaved at 121 °C (250 °F) for 60 min, cooled, inoculated with spore suspensions of test fungi, and incubated at about 24 °C (75 °F) in the dark for at least 15 d. After incubation, inoculum was dried in open Petri plates within a cabinet. Inoculum dried within 5 to 7 d 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 a commercial peat moss and perlite growing medium (Sunshine Mix 4, Aggregate Plus, Sungro Horticulture, Bellevue, Washington) at a concentration of 1:50 (w:w). The inoculum-growing medium mixture was placed into plastic containers ("dibble tubes" 115 mm^3), which had previously been sterilized by immersion in hot water (71 °C [160 °F] for 5 min).

Seeds of koa from a single family were nicked at their distal end with nail clippers to break dormancy, soaked in water for about 12 h, and sown into flats containing a 50:50 (v:v) mixture of vermiculite (Sta-Green Horticultural Vermiculite, St Louis, Missouri) and perlite (Redco II, North Hollywood, California). Flats were periodically watered and monitored for germination. Following germination, when radicles were approximately the same length as cotyledons, seedlings were carefully extracted from the flat and transplanted into the plastic containers containing the inoculum-growing medium

TABLE 1

Fusarium *isolates evaluated for pathogenicity on* Acacia koa *seedlings.*

Isolate number	*Fusarium* species	Isolation location
0421A	*F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas (Hyphomycetes)	Insect-predated koa seeds and seedpods
0421J	*F. subglutinans*	Insect-predated koa seeds and seedpods
0424E	*F. solani* (Mart.) Appel & Wollenw. (Hyphomycetes)	Stem of diseased koa seedling
0424I	*F. solani*	Stem of diseased koa seedling
0425K	*F. oxysporum* Schlechten.:Fr. (Hyphomycetes)	Roots of diseased koa seedling
0429K	*F. oxysporum*	Rhizosphere around roots of diseased seedling
0430B	*F. oxysporum*	Soil near roots of diseased koa seedling
0431B	*F. oxysporum*	Stem of diseased koa seedling
0433D	*F. oxysporum*	Roots of diseased koa seedling
0431J	*F. semitectum* Berk. & Rav. (Hyphomycetes)	Stem of diseased koa seedling

TABLE 2

Effects of selected Fusarium *isolates 120 d after germination on mean mortality, height, stem diameter, and root dry weight and subsequent vigor of 24 inoculated* Acacia koa *seedlings per isolate.*

Isolate number	*Fusarium* species	Mortality (%)	Height (cm)	Stem diameter (mm)	Root dry weight (g)	Vigor rating [z]
0421A	*F. subglutinans*	0	30.6 bc [y]	38 b	0.63 ab	0.91 dc
0421J	*F. subglutinans*	0	22.9 e	30 e	0.53 bc	0.96 bcd
0424E	*F. solani*	0	29.2 bc	33 de	0.52 bc	0.54 e
0424I	*F. solani*	0	23.8 e	30 e	0.48 cd	1.23 ab
0425K	*F. oxysporum*	4.2	24.3 de	35 cd	0.55 bc	0.79 de
0429K	*F. oxysporum*	4.2	35.6 a	42 a	0.73 a	0.59 e
0430B	*F. oxysporum*	0	27.2 cd	32 de	0.52 bc	1.22 abc
0431B	*F. oxysporum*	8.3	22.6 e	29 e	0.39 d	1.36 a
0433D	*F. oxysporum*	8.3	31.2 b	38 bc	0.61 ab	1.05 bcd
0431J	*F. semitectum*	25.0	28.3 bc	36 bc	0.52 bc	1.18 abc
—	Control	0	38.8 a	42 a	0.60 bc	0 f

[z] Average vigor rating at the end of the test; see text for descriptions.

[y] Within each column, means followed by the same letter are not significantly different ($P = 0.05$) using the Waller-Duncan K-ratio t test.

mixture. Following transplanting, seedlings were watered to activate inoculum. For each tested isolate, 4 replications of 6 seedlings each were evaluated. A fully replicated set of 24 seedlings was included as a control, which was transplanted into the peat moss and perlite growing medium 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 medium, and analyzed in the laboratory for root colonization by inoculated isolates. For this analysis, 10 root pieces, each approximately 5 mm in length, from each seedling were surface sterilized in a 1 part household bleach (5.25% aqueous sodium hypochlorite) to 10 parts water solution for 2 to 3 s; rinsed in sterile, distilled water; and placed on an agar medium selective for *Fusarium* spp. (Komada 1975). Plates were incubated under diurnal cycles of cool, fluorescent light at about 24 °C (75 °F) for 7 to 10 d. Emerging fungi were compared with inoculated isolates to determine whether they were the same morphological species.

At the end of the experiment (120 d), all surviving seedlings were examined for aboveground disease symptoms. Each seedling was rated for vigor based on severity of disease symptoms using the following numerical system: 0 = no disease symptoms and seedling appears healthy; 1 = seedling with < 50% of its crown with wilt or foliar chlorosis/necrosis symptoms; 2 = seedling with > 50% of its crown with wilt or foliar chlorosis/necrosis symptoms, but still alive; 3 = seedling near-dead (entire crown with disease symptoms). Seedling stem diameter just above the ground line and height from the ground line to the highest point of the main stem were measured on each surviving seedling. Seedling roots were washed thoroughly, surface sterilized as described previously, and analyzed for colonization by inoculated isolates as described above. Remaining roots were dried for at least 24 h at 100 °C (212 °F) and weighed. Means of seedling height, stem diameter, root weight, and average vigor rating were compared. Significant differences ($P = 0.05$) found among isolates were determined using the Waller-Duncan K-Ratio t test (SAS Institute 1982).

A numerical system was developed to quantitatively compare virulence among tested isolates using all measured parameters: seedling mortality, height, stem diameter, root dry weight, and vigor rating. This system assigned numbers for each parameter based on mean comparisons associated with each isolate, with lower numbers representing less fungal effects, that is, less mortality and greater seedling height, stem diameter, root dry weight, and subsequently higher vigor. Using this numerical system, *Fusarium* isolates were ranked on the basis of their overall virulence on koa seedlings.

RESULTS

Low levels of koa seedling mortality resulted from inoculations with most of the *Fusarium* isolates (Table 2). Significant differences ($P = 0.05$), however, were found among different isolates for the other measured parameters, including seedling height, stem diameter, root dry

TABLE 3

Comparative virulence of selected Fusarium *isolates on inoculated* Acacia koa *seedlings.*[z]

Isolate number	*Fusarium* species	Mortality	Height	Stem diameter	Root dry weight	Vigor	Virulence rating
Control	—	1	1	1	2	1	6 Nonpathogenic
0429K	*F. oxysporum*	2	1	1	1	2	7 Nonpathogenic
0421A	*F. subglutinans*	1	2	2	1	3	9 Low
0424E	*F. solani*	1	2	3	2	2	10 Low
0433D	*F. oxysporum*	3	2	2	1	3	11 Moderate
0425K	*F. oxysporum*	2	4	3	2	2	13 Moderate
0430B	*F. oxysporum*	1	3	3	2	4	13 Moderate
0431J	*F. semitectum*	4	2	2	2	4	14 Moderate
0421J	*F. subglutinans*	1	5	5	2	3	16 Moderate
0424I	*F. solani*	1	5	5	3	5	19 High
0431B	*F. oxysporum*	3	5	5	4	6	23 High
Maximum	—	4	5	5	4	6	24 High

[z] Numbers in table compare averages for each isolate; higher numbers reflect greater virulence.

weight, and average vigor rating. When all parameters were collated, isolates were ranked as to their relative virulence on inoculated seedlings (Table 3). This ranking indicated that the 5 tested isolates of *F. oxysporum* varied widely in their relative virulence, containing the most (0431B) and least (0429K) virulent of all evaluated isolates as well as 3 that were moderately virulent. Likewise, the 2 *F. solani* isolates exhibited relatively low (0424E) and high (0424I) virulence. One *F. subglutinans* isolate (0421A) was weakly virulent, whereas the other isolate of this species (0421J) was moderately virulent. The only tested *F. semitectum* isolate (0431J) was moderately virulent, although it was associated with the highest amount of seedling mortality (Table 2).

DISCUSSION

Fusarium isolates obtained from either diseased koa plants, soil adjacent to diseased plants, or seeds and seedpods varied widely in their virulence on inoculated koa seedlings under greenhouse conditions. This included several isolates of *F. oxysporum*, the putative cause of koa wilt and dieback disease. Apparently not all isolates of this *Fusarium* species are equally virulent on koa seedlings, even though they may be isolated from diseased plant material. This is not surprising because isolates morphologically classified as *F. oxysporum* likely comprise several different biological species based on their genetic differences (Gordon and Martyn 1997; Kistler 1997; Di Pietro and others 2003). Large numbers of *F. oxysporum* associated with either healthy or diseased plants are likely saprophytic (Gordon and Martyn 1997; Bao and others 2002; Lori and others 2004); a much smaller number are capable of eliciting disease (Bao and Lazarovits 2001; Roncero, and others 2003). Our results indicated that at least one tested isolate of *F. oxysporum* (0431B) was quite virulent on inoculated seedlings, whereas another isolate (0429K) was definitely saprophytic (Table 3). The former isolate was obtained from a diseased koa seedling, whereas the latter was isolated from rhizosphere soil around the roots of a diseased plant. We plan to retest both isolates in subsequent greenhouse inoculation trials to confirm their pathogenic or saprophytic behavior. The other tested *F. oxysporum* isolates were moderately virulent, indicating that these isolates were probably not aggressive pathogens, at least under our experimental conditions. These isolates may be facultative pathogens that require high levels of plant stress to cause disease (Nelson and others 1981; Bao and others 2002).

We have routinely isolated high levels of *F. solani* from diseased koa plants (James 2004). This species is most often isolated from the interior of stems, branches, and large roots of plants displaying crown koa wilt symptoms, particularly from tissues adjacent to typical interior greenish-charcoal staining that is often associated with diseased plants. *Fusarium solani* has also commonly been associated with infestation by black twig borers (*Xylosandrus compactus* Eichhoff [Coleoptera: Curculionidae]), insects that routinely attack diseased trees (Daehler and Dudley 2002). The 2 isolates evaluated in this test displayed either relatively high or relatively low virulence (Table 3). Roles of *F. solani* in koa disease etiology are currently unknown but may be important based on the relative frequency with which it is isolated from diseased plants. Further evaluation of its potential importance is warranted.

Fusarium subglutinans was generally isolated from insect-predated seeds and seedpods collected from koa trees displaying koa wilt symptoms (James 2004). This fungal species has previously been reported in Hawai'i on plants in the genus *Aglaonema* Schott (Araceae) (Uchida and Aragaki 1994). Isolates morphologically classified as this species may actually comprise several genetically distinct species (Nirenberg and O'Donnell 1998; O'Donnell and others 1998). For example, 2 isolates obtained from diseased koa plants morphologically classified as *F. subglutinans* were genetically identified as *F. sterilihyphosum* Britz, Marasas & Wingfield sp. nov. (Hyphomycetes) (O'Donnell 2005), a species previously described only on malformed mangos in South Africa (Britz and others 2002). The 2 *F. subglutinans* isolates we tested were either nonpathogenic or exhibited moderate virulence on koa seedlings. We suspect that this species (or species complex) may be associated with or vectored by seed-infesting insects and may not necessarily be important in disease etiology.

Fusarium semitectum is a relatively common fungal species within subtropical or tropical environments (Nelson and others 1981, 1983). This species can be pathogenic on several different plant species (Onyike and Nelson 1992; Satou and others 2001; Dhingra and others 2002; Bokshi and others 2003) and often produces mycotoxins that may adversely affect plants and animals (Abbas and others 1995; Jimenez and others 1997; Logrieco and others 2002). The one isolate we tested was rated as moderately virulent on koa seedlings, although a quarter of the seedlings inoculated with this isolate died during our study. This relatively high mortality as compared with other tested *Fusarium* isolates may have been because these seedlings were the least vigorous; these seedlings were the slowest to germinate and the last to be collected from germination trays. *Fusarium semitectum* is frequently isolated from diseased koa plants and its potential role in disease etiology warrants further investigation.

We were able to identify at least one *Fusarium* isolate (0431B) that may have some potential for screening koa families for resistance to the koa wilt disease; but overall our inoculation study indicated that *Fusarium* isolates obtained from diseased koa seedlings, adjacent soil, and seeds and seedpods are not necessarily pathogens. Five tested isolates of *F. oxysporum* showed a wide range of virulence on seedlings. This would indicate that both

pathogenic and nonpathogenic strains of this species, the putative cause of koa wilt, commonly colonize koa plants. Therefore, all isolates of *F. oxysporum* obtained from diseased koa plants are not necessarily the pathogen capable of eliciting koa wilt. Isolates will have to be genetically analyzed to locate potential markers associated with pathogenicity. Such markers may be effective to quickly and easily locate pathogenic strains from within fungal populations (Bao and others 2002; Lori and others 2004; Stewart and others 2004). Further pathogenicity tests, particularly with isolates of *F. oxysporum*, are planned to search for additional isolates that can be effectively used for resistance screening. When mixtures of pathogenic strains are available, we should be successful in screening for disease resistance.

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