FOREST PEST MANAGEMENT

PATHOGENICITY OF FUSARIUM FROM FOREST SEEDLING NURSERIES ON DOUGLAS-FIR AND PONDEROSA PINE SEEDLINGS

by

R. L. JAMES, E. P. MILITANTE,
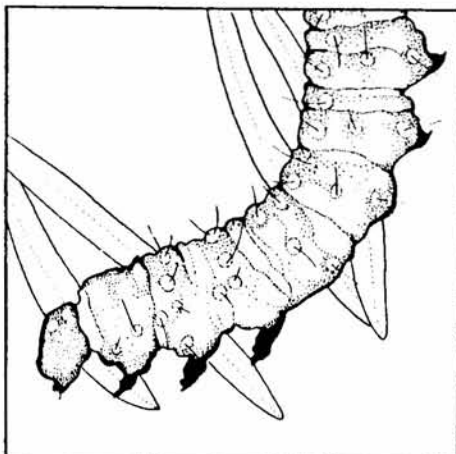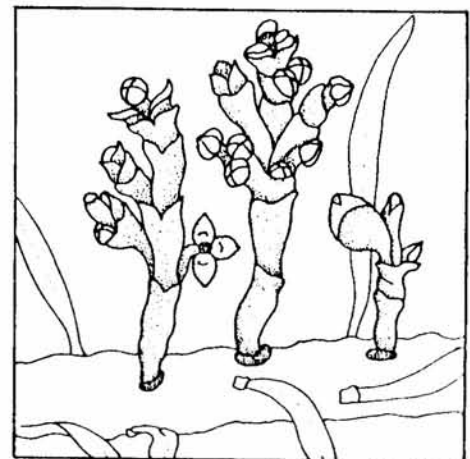
J. Y. WOO and C. J. GILLIGAN
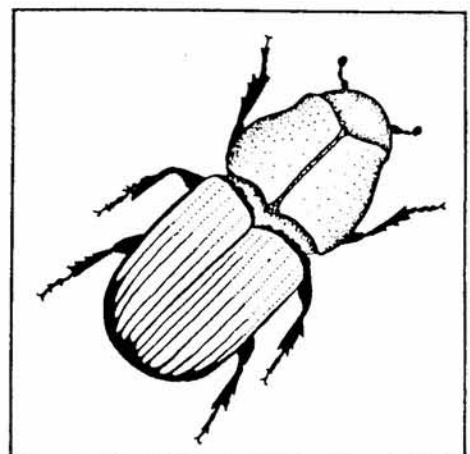
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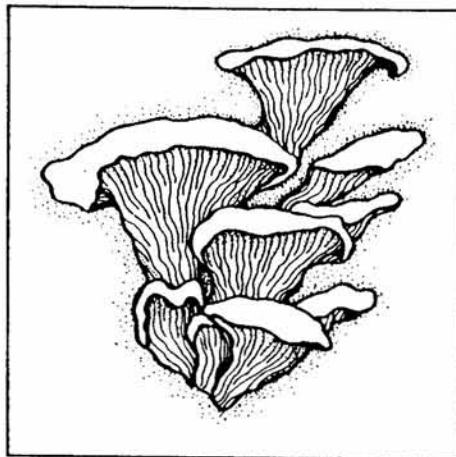
by

R. L. James, Plant Pathologist,
Cooperative Forestry and Pest Management

E. P. Militante, Graduate Research Assistant,
University of Idaho[1]

J. Y. Woo, Research Plant Pathologist,
Intermountain Forest and Range Experiment Station

C. J. Gilligan, Laboratory Technician,
Cooperative Forestry and Pest Management

[1]Present address: Professor, Forest Biological Science Department,
University of Philippines, College of Forestry, Laguna, Philippines 3720

ABSTRACT

Eighteen isolates of _Fusarium_ comprising four species (_F. oxysporum_, _F. avenaceum_, _F. acuminatum_, and _F. sambucinum_) isolated from diseased conifer seedlings from nurseries were tested for pathogenicity on Douglas-fir germlings. Three of the most pathogenic _F. oxysporum_ isolates on Douglas-fir germlings were also tested on ponderosa pine germlings and older Douglas-fir seedlings; three of the most pathogenic _F. sambucinum_ isolates on Douglas-fir germlings were likewise tested on older Douglas-fir seedlings. Most _F. oxysporum_ isolates and all the other fusaria were highly pathogenic to germlings. Level of virulence on germlings was best measured by rate of infection and tissue degradation. All inoculated older Douglas-fir seedlings became infected, although foliar disease symptoms were rare. Based on extent of root system colonization, isolates of _F. oxysporum_ were generally more pathogenic to older Douglas-fir seedlings than _F. sambucinum_. These tests confirm the wide range of pathogenicity of fusaria commonly isolated from diseased seedlings.

INTRODUCTION

Fusarium spp. are frequently isolated from the roots of conifer seedlings in nurseries. They may be associated with a wide range of disease symptoms (James 1985b) or with healthy appearing seedlings (Bloomberg 1966; James and Gilligan 1984). Therefore, occurrence of _Fusarium_ on seedlings does not necessarily mean that it is pathogenic. Factors mediating pathogenic activity of these fungi involve both host responses and environmental effects.

To more clearly define the role of _Fusarium_ as a conifer seedling pathogen, standard tests evaluating pathogenicity are needed. This report summarizes two tests used to quantify pathogenicty and elucidate levels of virulence of several _Fusarium_ isolates obtained from conifer seedlings in nurseries.

MATERIALS AND METHODS

Isolates of _Fusarium_ tested in this study were obtained mostly from diseased conifer seedlings from several northern Rocky Mountain forest tree nurseries (Table 1). Eighteen isolates were evaluated on germlings in aseptic test tube inoculations and six of the most pathogenic were further tested on older and larger potted seedlings.

Test Tube Inoculations--Douglas-fir (_Pseudotsuga menziesii_ (Mirb.) Franco) and ponderosa pine (_Pinus ponderosa_ Laws.) seed were surface sterilized in 40 percent bleach solution (2.1 percent aqueous sodium hypochlorite) for 10 minutes and washed thoroughly under running tap water for 4 days in order to remove contaminating fungi. Seed were then stratified at 0 to 3°C for 7 days. After stratification, seeds were sown in plastic petri dishes lined with four layers of sterile, moist filter paper and incubated at about 21°C under an 8-hour light and 16-hour darkness regime. Germlings were used for inoculations when they were 10 to 15 mm in length (about 3 to 5 days old).

Table 1.--Description of _Fusarium_ isolates tested for pathogenicity on Douglas-fir and ponderosa pine seedlings.

Isolate designation	Species[1]	Host Species[2]	Host Tissue	Nursery
82-59F	FOXY	DF	Seed	USDA For. Serv., Coeur d'Alene, ID
83-42A	FOXY	DF	Roots	Montana State, Missoula, MT
83-59A	FOXY	DF	Seed	Montana State, Missoula, MT
83-71-10	FOXY	WF	Roots	Fantasy Farms, Peck, ID
83-73	FOXY	DF	Roots	USDA For. Serv., Coeur d'Alene, ID
83-74	FOXY	DF	Roots	USDA For. Serv., Coeur d'Alene, ID
84-11	FOXY	DF	Roots	Montana State, Missoula, MT
84-21	FOXY	ES	Roots	Alpine, Kalispell, MT
84-22-D2	FOXY	-	Soil Mix[3]	Alpine, Kalispell, MT
82-56G	FSAM	RO	Stems	Montana State, Missoula, MT
82-57B	FAVE	DF	Seed	USDA For. Serv., Coeur d'Alene, ID
73-1F	FACU	PP	Needles	Montana State, Missoula, MT
83-51A	FSAM	PP	Roots	Dan Day, Kalispell, MT
83-67 #1	FSAM	LP	Roots	Alpine, Kalispell, MT
83-91A #1	FSAM	PP	Stems	USDA For. Serv., Albuquerque, NM
84-13-B3	FACU	-	Soil Mix[3]	Montana State, Missoula, MT
84-21 #1	FACU	ES	Roots	Alpine, Kalispell, MT
84-22 D3	FAVE	-	Soil Mix[3]	Alpine, Kalispell, MT

[1]FOXY = _Fusarium oxysporum_; FAVE = _Fusarium avenaceum_;
 FACU - _Fusarium acuminatum_; FSAM = _Fusarium sambucinum_

[2]DF = Douglas-fir; WF = white fir; ES = Engelmann spruce; RO = Russian-olive;
 PP = ponderosa pine; LP = lodgepole pine.

[3]Standard peat-vermiculite soil mix used to grow containerized seedlings;
 samples were from mixes within which diseased conifer seedlings were growing.

Test tubes for inoculations were prepared by placing two layers of folded filter paper within each tube (to provide a platform away from the bottom of tubes to support germlings and inoculum), followed by 6 ml sterilized, distilled water; covering with a foam plug; and sterilizing by autoclaving. Within each sterilized test tube, a plug of inoculum about 5 mm in diameter, (1-week-old culture grown on potato dextrose agar (PDA)) was aseptically placed on the folded filter paper. Control treatments consisted of test tubes with agar plugs only. Germlings were aseptically placed in test tubes so that their roots were in contact with the inoculum. Each isolate was replicated 10 times. Inoculated and uninoculated (control) germlings were incubated at 21°C under a diurnal regime of 8 hours of light and 16 hours of darkness.

Germlings were examined periodically for disease symptoms. The experiment was terminated after 22 days at which time isolations were made from the roots of germlings. Isolates obtained were compared with the original inoculum, which was maintained on PDA slants, to determine if they were the same.

All isolates were tested on Douglas-fir germlings. But because of poor ponderosa pine seed germination, only three of the most pathogenic F. oxysporum Schlect. isolates on Douglas-fir germlings (82-59F, 83-74, 84-21) were tested on pine germlings.

Douglas-fir Pot Inoculations--Six of the more pathogenic isolates from the test tube inoculations (82-56G; 82-59F; 83-42A; 83-51A; 83-67 #1; 83-73) were tested on containerized Douglas-fir seedlings by inoculating the potting medium. These seedlings were 8 months old and had been grown in styroblock containers.

Inoculum consisted of a peat-perlite-Czapek agar mixture. Inoculum was prepared by mixing 200 ml Czapek agar solution with 1,500 mg peat-perlite mixture and autoclaving for 30 minutes. When cooled, 1,200 ml of this agar mixture was combined with the contents of three culture tubes of the test fungus (7-day-old cultures) which had been macerated in 300 ml sterile water. After inoculation, the mixture was incubated for 2 weeks at 24°C. Following incubation, 200 ml of the inoculum mixture was combined with 1,200 ml of a sterilized peat-vermiculite growth medium placed in pots, into which test seedlings were transplanted. Controls consisted of adding noninoculated Czapek-peat-perlite mixtures to sterilized peat vermiculite. Care was taken to wash seedling root systems thoroughly and avoid wounding during transplanting. Twelve seedlings were inoculated with each test fungus. Inoculated seedlings were maintained on greenhouse benches and checked periodically for symptoms.

Eleven months after inoculation, all seedlings were analyzed for infection. A five-class rating system was formulated for estimating severity of foliar symptoms on inoculated seedlings (Table 2). Procedures for quantitatively estimating extent of root system colonization by Fusarium are summarized in Table 3. These procedures allowed a fairly accurate estimate of the degree of virulence as measured by level of root system colonization. Isolates recovered from roots were compared with the original inoculum to determine if they were the same.

Table 2.--Foliar symptom classifications of Douglas-fir seedlings inoculated with Fusarium.

Class	Symptoms[1]
1	Greater than 80% of foliage green and healthy
2	50-80% of foliage green
3	30-50% of foliage green
4	10-20% of foliage green
5	Less than 10% of foliage green

[1]Nongreen foliage was chlorotic or necrotic.

Table 3.--Procedures for estimating amount of infection of Douglas-fir
seedlings with Fusarium.

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1. Wash entire root system under tap water to remove loose soil
   adhering to roots.

2. Surface sterilize root system in a bath of 4.0 percent bleach
   (0.21 percent aqueous sodium hypochlorite) for 4 minutes.

3. Rinse sterilized root system thoroughly under tap water to
   remove excess bleach.

4. Randomly select 10 lateral roots and aseptically cut them
   from the main tap root.

5. Cut tips (about 0.5 cm in length) from the 10 selected roots
   and aseptically place on selective medium (Komada 1975).

6. Cut a piece from the top of selected roots (where they join
   the main tap root) and aseptically place on selective medium.

7. Incubate plates for 7 days at about 24°C under a 12-hour
   diurnal light-darkness regime.

8. Count the number of root pieces infected with Fusarium and
   calculate: (a) percent of roots, (b) percent of root tips
   and (c) percent of root junctions infected.

Histological Studies--Infected germlings from the test tube inoculation
experiment were sectioned where the main tap root came into contact with
inoculum. Four of the isolates considered most pathogenic (82-59F, 83-42A,
83-74, and 84-21) because they killed all germlings within 22 days of
inoculation, were evaluated. Tissues were fixed in formalin-acetic acid
alcohol (FAA), dehydrated in tertiary butyl alcohol, and embedded in paraffin.
After embedding, tissues were cut with a rotary microtome at 12 u and stained
with Feulgens and fast green. Sections were examined under the light
microscope (100-450X) for hyphal interactions with host cells.

RESULTS

Test Tube Inoculations--Six of the nine isolates of F. oxysporum were
considered highly pathogenic (Table 4), i.e., they killed all Douglas-fir
germlings within 22 days of inoculation. Most of these isolates initially
penetrated roots and began tissue degradation within 2 to 3 days. Less
pathogenic isolates killed only 40 to 70 percent of the germlings and usually
took longer (12 to 15 days) to infect and degrade host tissues than highly
pathogenic isolates. The three F. oxysporum isolates tested against ponderosa
pine germlings (82-59F, 83-74, 84-21) were also pathogenic to this species.
Inoculated pine germlings were invaded quickly and killed within 22 days.
Control germlings were not killed.

Table 4.--Susceptibility of Douglas-fir germlings to selected
isolates of Fusarium oxysporum.

Isolate[1]	Percent germlings killed after 22 days[2]
82-59F	100
83-42A	100
83-73	100
83-74	100
84-21	100
84-22D3	100
83-59A	40
83-71-10	60
84-11	70
Controls	0

[1] See Table 1 for isolate description.

[2] Most pathogenic isolates (100 percent of germlings killed)
caused initial infection and degradation of roots within 2
to 3 days. The less pathogenic isolates took longer (12 to
15 days) for infection and degradation to occur.

All the other Fusarium species (F. avenaceum, F. acuminatum, F. sambucinum)
were highly pathogenic, invading and killing Douglas-fir germlings within 4 to
5 days of inoculation.

Douglas-fir Pot Inoculation--Results of inoculating containerized Douglas-fir
seedlings with six of the most pathogenic isolates tested on germlings are
summarized in Table 5. All seedlings became infected with the inoculated
isolates. In general, F. oxysporum isolates colonized a greater portion of the
seedling root system than the F. sambucinum isolates. Low average symptom
ratings indicated that many infected seedlings lacked noticeable foliar
symptoms. Relationships between amount of root system infection and intensity
of foliar symptoms were not apparent in this study. Several noninoculated
control seedlings became infected with Fusarium, although amount of root
infection was low. Seedlings selected for inoculation probably had a low level
of "background" infection. Since the fungi reisolated from inoculated
seedlings generally corresponded to those inoculated, apparently these
"background" fusaria were readily replaced by the inoculated isolates.

Histological Studies--Microscopic examinations of sections of primary roots
where they came into contact with inoculum in germling test tube inoculations
showed that invasion of roots by F. oxysporum was very rapid. Twenty-four
hours after inoculation the fungus had entered the roots and in some samples
had spread to cells on the outer edges of the vascular or central root
cylinders (figure 1). Three days after inoculation, most isolates had spread
to all root cells and some cortex cells were beginning to deteriorate (figure
2). Six days after inoculation all root cells were completely invaded by F.
oxysporum and usually extensively deteriorated (figure 3).

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Table 5.--Pathogenicity of selected Fusarium isolates to containerized
Douglas-fir seedlings.

Isolate	Species	Average symptom rating[1]	Percent infection			
			Seedlings	Root tips	Root joints	Root system[2]
82-59F	F. oxysporum	1.9	100.0	90.8	77.5	84.2A
83-42A	F. oxysporum	1.7	100.0	95.0	77.5	86.3A
83-73	F. oxysporum	1.8	100.0	99.2	91.7	95.4A
82-56G	F. sambucinum	1.4	100.0	75.8	61.7	68.8B
83-51A	F. sambucinum	2.0	100.0	63.3	40.8	52.1B
83-67 #1	F. sambucinum	1.2	100.0	75.8	59.2	57.5B
Control	--	1.2	58.3	19.2	8.3	13.8C

[1] See Table 2 for description.

[2] Estimate of the overall percentage of the root system infected
(average of root tips and root joints). Values followed by the same
capital letter are not significantly different (P = 0.05) using Duncan's
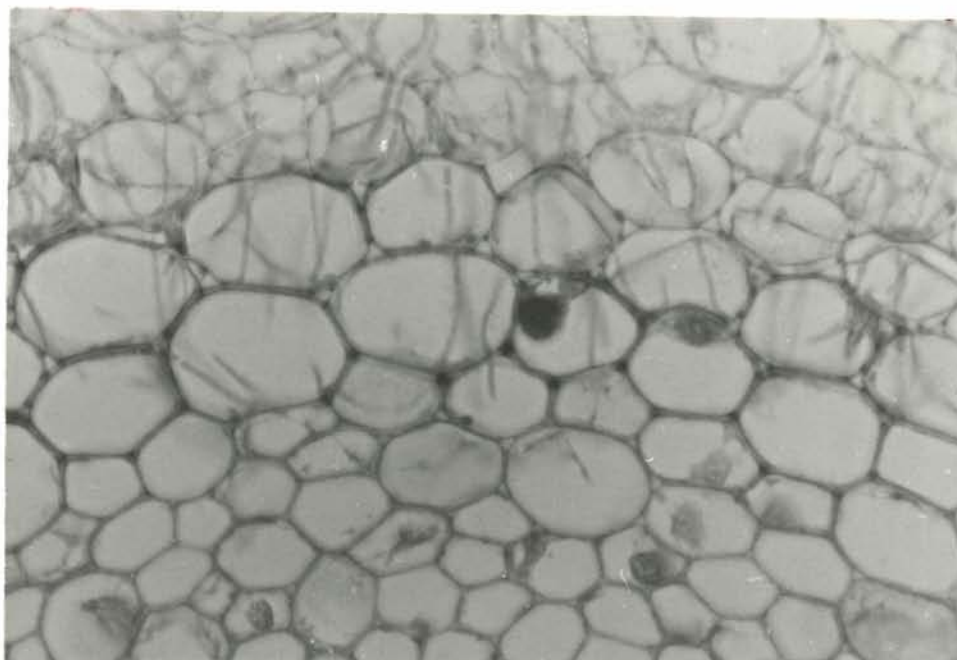Multiple Comparison Test.

Figure 1.--Taproot cross-section of a Douglas-fir germling invaded by
Fusarium oxysporum (isolate 82-59F) 24 hours after inoculation
(X250). Root epidermis is at the top and the central vascular
cylinder is at the bottom. Fungal hyphae had readily colonized
cortical cells between the epidermis and central vascular
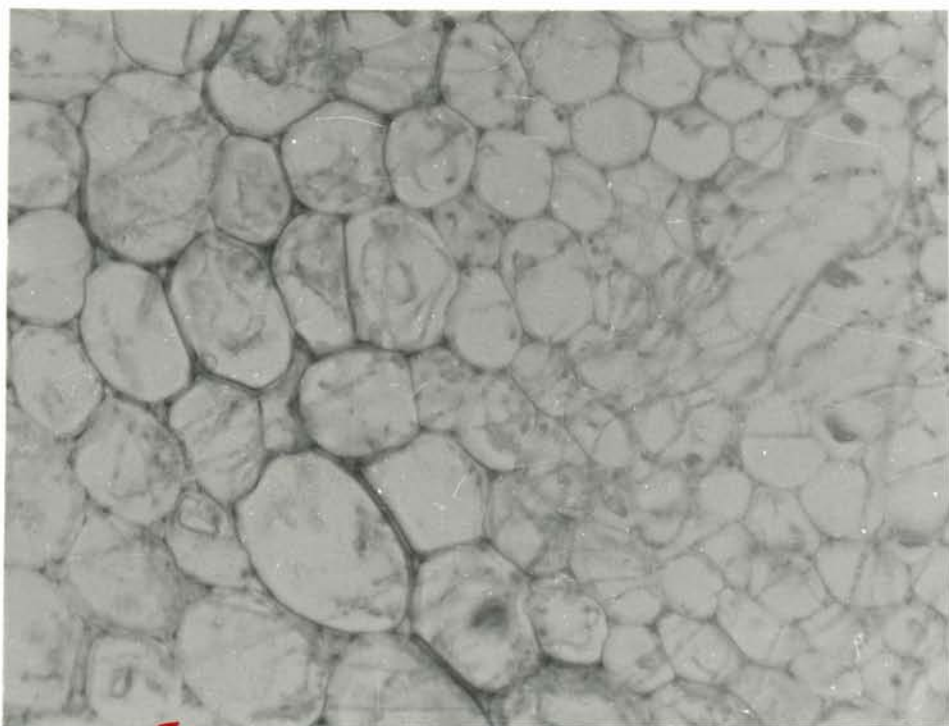cylinder.

Figure 2.--Taproot cross-section of a Douglas-fir germling invaded by
Fusarium oxysporum (isolate 83-74) 72 hours after inoculation
(X250). Root epidermis is to the left and the central vascular
cylinder is to the right. Fungal hyphae had penetrated to the
vascular cylinder and a few cortical cells had deteriorated.
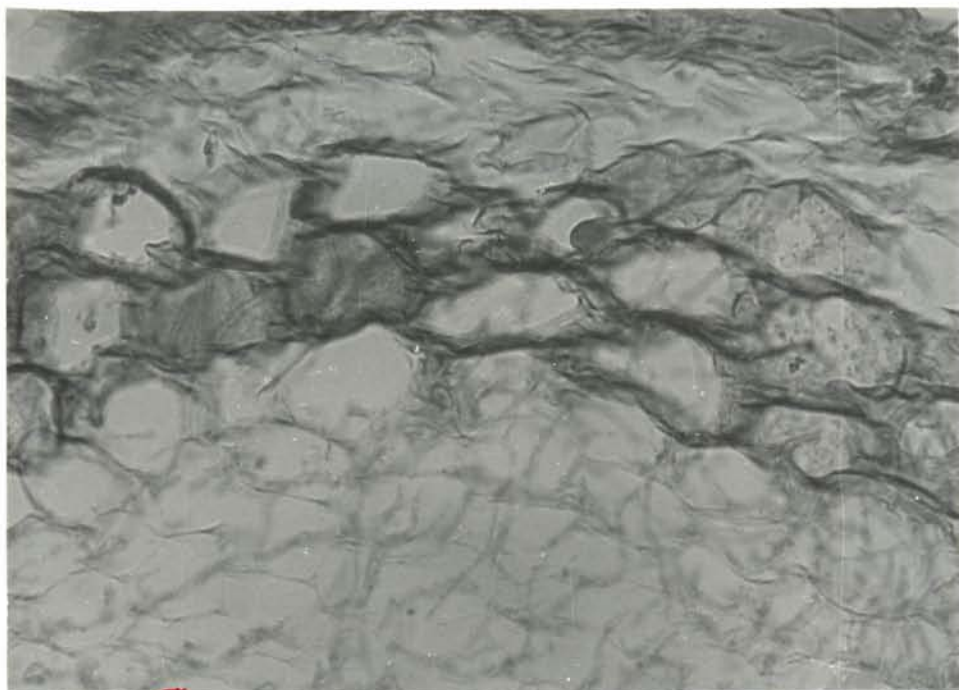

Figure 3.--Taproot cross-section of a Douglas-fir germling invaded by
Fusarium oxysporum (isolate 84-21) 144 hours after inoculation
(X250). Root epidermis is at the top and the central vascular
cylinder is at the bottom. All root cells are completely
colonized by fungal hyphae and extensive root deterioration is
evident.

DISCUSSION

Our studies confirmed pathogenic variability of individual _Fusarium_ species
from conifer seedlings (Armstrong et al. 1940; Gordon 1965; James and Gilligan
1984; Wellman and Blaisdell 1941). Pathogenic variability may be related to
differential host responses (i.e., host resistance) and inherent genetics of
fungal pathogenicity (Gordon 1965). Several workers (Armstrong et al. 1940;
Harvey 1929; Wellman and Blaisdell 1941) have attempted to correlate level of
pathogenicity with cultural characteristics such as growth rate and tendency to
produce abundant aerial mycelium. Our experience thus far indicates that
virulent isolates cannot consistently be predicted on the basis of stable
cultural characteristics. Inoculation tests on conifer hosts are apparently
required.

Inoculations of young conifer germlings in sterile test tubes have previously
been successful in differentiating pathogenic _Fusarium_ isolates (James 1985c;
Tint 1945). Advantages of this system include rapidity of test results and the
limited resources needed to conduct such tests. However, our results on
Douglas-fir and ponderosa pine indicated that only the least pathogenic
isolates were differentiated in germling inoculation tests. Although
pathogenic and saprophytic isolates can usually be separated in germling tests,
the level of virulence may be obscured because of large inoculum potential.
Therefore, rate of infection and tissue degradation are probably better
indicators of level of virulence than assessment of mortality rates. We would
expect the more virulent isolates to infect and colonize germlings more rapidly
than less virulent isolates. On the basis of rapidity of infection and tissue
degradation, we were able to identify the most virulent _F. oxysporum_ isolates.
However, germling tests were unable to differentiate isolates of the other
Fusarium species tested (_F. avenaceum_, _F. acuminatum_, _F. sambucinum_). All of
these species rapidly invaded and killed germlings.

To more closely simulate disease conditions expected in a nursery, inoculation
tests on larger seedlings were necessary. Although such tests take longer to
assess, they often provide a better basis for separating virulent and
nonvirulent fusaria. Ideally, inoculations would consistently separate
pathogenic from saprophytic isolates on the basis of level of seedling
mortality and symptom expression. However, in our tests most infected
seedlings lacked foliar disease symptoms even though roots of all inoculated
seedlings became colonized with test isolates. Previous work (Bloomberg 1966)
indicated that _Fusarium_ often invades conifer seedling roots without eliciting
host responses such as foliar symptoms. Other studies (James 1984a; James
1984b) also indicated that seedlings may be infected for extended periods
without displaying symptoms. However, during periods of seedling stress, such
as limiting nutrients and water during bud set in container operations, fusaria
may become aggressive and cause seedling decline with associated symptoms
(James 1985b). In our studies, inoculated seedlings were maintained for 11
months because they generally lacked disease symptoms. However, we suspect
that seedling roots probably became infected shortly after inoculation.
Abundance of nutrients and moisture added to test seedlings may have reduced
symptom expression, or the isolates tested may not have been aggressive enough
to consistently overcome host defenses. Therefore, we based our assessment of
virulence mostly on extent of root system colonization. The more aggressive
isolates would probably colonize a greater portion of the root system.
Isolates classified as virulent on the basis of root system colonization did

not necessarily cause the most severe disease symptoms; i.e., level of root colonization was not related to amount of foliar symptom production. On the basis of root system colonization, the F. oxysporum isolates tested were more pathogenic than the F. sambucinum isolates.

Some outplanted seedlings infected with Fusarium may be considered "healthy" because of lack of disease symptoms. Smith (1967) showed that F. oxysporum on seedling roots is readily replaced by other soil fungi when infected seedlings are outplanted on forest soils. However, other work (James 1985a) indicates that if much of the root system is infected, stress induced by storage or outplanting may be sufficient to cause extensive mortality of planted seedlings during the first growing season.

Our results indicated that most of the F. oxysporum isolates tested were pathogenic to conifer seedlings. This confirms other work (Gordon 1965; James and Gilligan 1984; Matuo and Chiba 1966), although levels of virulence may vary widely. The other tested fusaria were originally classified as F. "roseum" based on older taxonomic schemes of Snyder and Hansen (Toussoun and Nelson 1968). This grouping was unsatisfactory because many of the organisms classified as the same species behaved quite differently. Therefore, more modern and acceptable taxonomic schemes (Booth 1971; Gerlach and Nirenberg 1982) were used to separate isolates into three species: F. avenaceum, F. acuminatum, and F. sambucinum. In the past, many F. "roseum" isolates have been considered saprophytic on conifer seedlings (Buxton et al. 1962; Vaartaja and Cram 1956). However, other work (James and Gilligan 1984; Matuo and Chiba 1966; Morgan 1983) indicates that F. "roseum" may be very pathogenic to conifer seedlings at times. In our tests, the F. "roseum" isolates were very pathogenic on germlings, but less so on older Douglas-fir seedlings.

Another problem arises because of common changes in Fusarium isolates due to mutations. Such changes include colony morphology and probably affect pathogenicity. Therefore, level of pathogenicity may change over time depending on the amount of isolate mutation that occurs.

In conclusion, our work indicated that the fusaria commonly associated with conifer seedling diseases includes organisms that vary in their ability to incite diseases. Further work is needed to elucidate roles of separate species when several fusaria are isolated from individual diseased plants. Organism succession or synergistic relationships among various fungi may be involved. Continued research should receive high priority because of the widespread impact of Fusarium-associated diseases in conifer nurseries and the relatively high value of affected hosts.

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