Infection of Austrian, Scots, and Ponderosa Pines by Diplodia pinea

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ABSTRACT

Spores of *Diplodia pinea* were disseminated from March to November in eastern Nebraska. Large numbers of spores were disseminated only during periods of rainfall. Spores germinated on water agar at temperatures ranging from 12 to 36 C. Per cent germination was highest at 24 C, and germ tube growth was maximum at 28 C. Spores started to germinate within 1.5 hr at 26 C; over 75% germinated within 2 hr.

Fluorescent-labeled spores, plastic prints of needle surfaces, and microtome sections of inoculated needles were used in establishing that the pathogen penetrates needles of Austrian pine (*Pinus nigra*), Scots pine (*P. sylvestris*), and ponderosa pine (*P. ponderosa*) through stomata. Some germ tubes on needle surfaces grew directly toward and entered stomatal pits, but most grew randomly. Needles inoculated with fluorescent-labeled spores contained masses of fluorescent hyphae in stomatal pits. Hyphae from these masses grew through stomata and into the mesophyll. Direct penetration of the epidermis was never observed.

Incubation for 12 hr in growth chambers at 100% relative humidity and 24 C was sufficient for infection of uninjured Austrian, Scots, and ponderosa pine needles. Initial symptoms, consisting of small, tan to reddish-brown lesions on the lower portion of young needles, appeared within 3 days after inoculation on Austrian and Scots pines and within 4 days on ponderosa pine. Needle discoloration and necrosis developed rapidly. Inoculations of 10-year-old trees in eastern Nebraska revealed that young shoots of all three species were highly susceptible to infection from late April until mid-June; previous years' needles were not susceptible. Phytopathology 61:409-414.

Diplodia pinea (Desm.) Kickx seriously damages numerous pine species in several areas of the world (5, 12, 13). In recent years, the pathogen has caused extensive damage to exotic pines in the central Great Plains (8).

The pathogen causes yellowing and necrosis of the current year's needles, and often kills entire shoots early in the growing season. Pycnidia develop near the base of needles and on leaf sheaths. Attached cones may also become infected; pycnidia are often abundant on cone scales. Severe infection year after year retards growth and may even kill trees (8, 14).

Bancroft (1) and Purnell (9) reported that only injured tissue was susceptible to infection. Waterman (14) reported, however, that wounding favored, but was not required for, infection of developing needles and elongating shoots. Abundant spores, high humidity, and temperatures near 28 C also favor infection (4, 9, 14). The time of year and conditions under which spores are disseminated are not described in the literature, although rain has been suggested as a means of spore dissemination (3, 4). Also, little is known about the histology of the early stages of the infection process. The presence of short, chlorotic needles on infected shoots indicates that infection occurs early in the growing season. However, the time of initial infection and the importance of late-season infection have not been determined.

Peterson & Wysong (8) reduced infection by approximately 50% with Bordeaux mixture applied in mid-May, mid-June, and mid-July. Additional information was needed, however, for the development of a more effective and economical control program.

The objectives of this research were to determine (i) when *Diplodia pinea* spores are disseminated; (ii) the effect of rain on spore dissemination; (iii) the influence of temperature on spore germination and germ tube development; (iv) the site and mode of needle penetration by the pathogen; (v) the time required at 100% relative humidity for infection to occur; and (vi) the susceptibility of needles at various times during the growing season.

MATERIALS AND METHODS.—*Spore dissemination.*—Petrolatum-coated microscope slides were attached beneath naturally infected pine shoots from 9 March to 6 November 1968, and from 21 April to 10 November 1969. The slides were collected periodically, and 195 mm^2/slide was scanned for *Diplodia pinea* spores. The amount of rainfall in the immediate vicinity was recorded.

Spore germination and needle penetration.—To determine the effect of temperature on spore germination and germ tube growth, we incubated spores produced in culture in the dark on 2% water agar at temperatures ranging from 8 to 40 C at 4-degree intervals. The spores were killed with 0.1% $HgCl_2$ after 4-hr incubation.

To determine the time required for germination and the rate of germ tube growth, we incubated spores on water agar and in distilled water at 26 C. After 0, 1, 2, 3, 4, 5, and 6 hr, the spores were killed with 0.1% $HgCl_2$.

In both tests, the percentage germination of 100 spores and the mean germ tube length of 30 spores were determined for each of three replications.

The early stages of the infection process were studied through histological examination of needles from Austrian pine (*Pinus nigra* Arnold), Scots pine (*P. sylvestris* L.), and ponderosa pine (*P. ponderosa* Laws.). Plastic leaf prints (7) were used to observe spore germination and hyphal growth on needle surfaces. Fluorescent labeling (6, 11) was used to determine the site and mode of penetration of pine needles by *D. pinea*.

Spores from single-spore isolates or from naturally infected pine cones were suspended in distilled water and used as inoculum. The suspensions were divided in half and placed in centrifuge tubes, and volumes adjusted to 12.5 ml. To one tube, 0.5 ml of a 1.0% solution of 4, 4′-bis 4-anilino-6-bis (2-hydroxyethyl) amino-*s*-triazin-2-ylamino-2,2′-stilbene-disulfonic acid was added. Ten min later, the suspensions were centrifuged at 1,100 *g* for 10 min. The liquid was decanted and the spores were resuspended in 13 ml of water. The spores were washed twice in this manner to remove excess brightener. Spore concentrations were determined with a hemacytometer.

A medicine dropper containing approximately 7.5×10^5 spores in 1 ml of water was used to inoculate about 50 needles on a shoot with unlabeled or fluorescent-labeled spores. The medicine dropper was positioned so that the needles of a fascicle were inside the dropper. The dropper was then removed, leaving a fairly uniform spore distribution on the needles. An atomizer was used to inoculate individual shoots on a tree. Inoculated plants were placed in a chamber at 26 C and 100% relative humidity. Needles were collected 4 hr after inoculation and daily until the needles became chlorotic.

Plastic prints of needle surfaces were made 4 hr after the needles had been inoculated with unlabeled or fluorescent-labeled spores. Needles were dipped in a solution of 4% cellulose acetate in acetone. The solution on the needles was allowed to dry, and the needles were exposed to acetone vapor until the white film became transparent. The film was then stripped from the needles and placed on a microscope slide containing a small amount of cotton blue in a drop of water. The leaf prints were observed microscopically, and areas containing germinating spores were photographed on Kodak 35 mm Panatomic X film.

Needles inoculated with labeled spores were examined with a Zeiss fluorescence microscope, and segments containing germinating spores were photographed. These segments were then fixed in FAA, dehydrated in *n*-butanol series (10), embedded in paraffin, sectioned at 10 or 14 μ on a rotary microtome, and affixed to slides. The paraffin was removed and the sections were hydrated and then mounted in phosphate-buffered glycerine. Observations were made with the fluorescence microscope, and locations of fluorescent hyphae were recorded.

Since hyphae inside the needle did not fluoresce brightly, staining was required to obtain photomicrographs. Slides containing fluorescent hyphae were placed in water, and the cover slips removed. The sections were dehydrated to 50% ethanol, stained in Pianeze 111-B (15) for 45 min, washed in 50 ml of 95% ethanol containing three drops of 1 N HCl, rinsed with clove oil, washed in three changes of xylene, and mounted in Fisher Permount. The fluorescent hyphae could be identified after staining, since their locations on the slide had been recorded. Photomicrographs were taken of areas containing fluorescent-labeled hyphae.

Inoculations in the greenhouse.—To determine the time required at 100% relative humidity for infection to occur, shoots of 1-year-old Austrian, Scots, and ponderosa pines were inoculated with spores from naturally infected pine cones. The inoculum was suspended in water and brushed onto the young shoots. Three inoculated trees of each species were placed in a 24-C chamber at 100% relative humidity for 0, 12, 24, 36, and 48 hr. Three noninoculated trees of each species were placed in the chamber for 2 days. After removal from the chamber, the trees were placed in a greenhouse at 40-60% relative humidity and a night temperature of 24 C. The young needles, which were approx 1 cm long, were examined daily for disease symptoms, and the number of infected shoots was determined.

Sixteen days after inoculation, an effort was made to reisolate the fungus from inoculated shoots. The shoots were swabbed with 50% ethanol, then air-dried. Sections of the shoots were aseptically transferred to potato-dextrose agar and placed in a lighted incubator at 28 C. The fungus was identified on the basis of colony, pycnidia, and spore characteristics.

Inoculations in the field.—To determine the time of initial infection and the importance of late-season infection, Austrian, Scots, and ponderosa pine shoots were inoculated periodically from 28 April to 16 September 1969, with spore suspensions from 3-week-old cultures of *D. pinea*. Three shoots on each of three 10-year-old trees of each species were inoculated. The lengths of five 1969 needles on each inoculated shoot were measured, and the inoculum was brushed onto the uninjured 1968 and 1969 needles. The inoculated needles were enclosed in a polyethylene bag containing a moist paper towel to maintain 100% relative humidity. A paper sack was placed over the bagged needles to prevent damage by excessive heat. Needles were uncovered 4 days after inoculation, and on the 7th day were sprayed with Bordeaux mixture to halt further penetration by the pathogen. Control shoots were treated in a similar manner, except that inoculum was excluded. The number of current-year needles out of 50 showing characteristic disease symptoms was determined for each shoot, and attempts were made to reisolate the fungus.

Growth and sporulation in culture.—To determine the effect of temperature on growth, a single-spore isolate of the fungus was incubated on potato-dextrose

agar at temperatures from 8 to 40 C at 4-degree intervals. The colony diameter of each of three replications was measured after 24, 48, and 72 hr.

Two isolates of the fungus were incubated in the dark and under six 15-w fluorescent bulbs on potato-dextrose agar, V-8 agar, malt agar, pine needle extract agar, and malt extract agar at 28 C. The diameter of each of three colonies was measured after 24, 48, and 72 hr. The number of pycnidia per plate was determined 10 days after inoculation.

RESULTS.—*Spore dissemination.—Diplodia pinea* spores were trapped during the exposure periods from March to October 1968 and from April to November 1969 (Table 1). Large numbers of spores were disseminated only during periods of rainfall.

Spore germination and needle penetration.—Diplodia pinea spores germinated on water agar at temperatures from 12 to 36 C. At 24 C, the optimum temperature for germination, 95% of the spores germinated after 4-hr incubation. At the optimum temperature for germ tube growth, 28 C, the mean germ tube length was 245 μ. Per cent germination did not vary greatly in the temperature range of 20 to 36 C. The rate of germ tube growth decreased sharply above and below 28 C.

Spores germinated rapidly on water agar at 26 C. Germ tubes were first observed after 1.5 hr incubation; after 2 hr, 77% of the spores had germinated, and within 3 hr, 87% had germinated. Spores germinated as well in distilled water as on water agar.

TABLE 1. *Diplodia pinea* spores trapped on microscope slides attached to Austrian pine shoots in 1969

Date exposure began	Days exposed	Rainfall during exposure	Spore count[a]
	no.	*inches*	*no.*
April 21	7	0.19	131
28	9	0.12	190
May 6	8	1.31	73
14	7	2.25	198
21	7	0.16	114
28	6	0.00	3
June 3	7	0.00	1
10	7	2.25	598
17	7	0.74	394
24	13	2.70	25
July 7	7	1.02	387
14	7	0.52	266
21	10	0.24	100
31	7	0.11	126
Aug. 7	7	0.00	2
14	7	0.95	922
21	7	0.00	9
28	7	0.80	211
Sept. 4	7	0.85	175
11	7	0.00	1
18	7	0.00	1
25	7	0.02	2
Oct. 2	7	0.06	5
9	7	1.85	165
16	11	0.46	40
27	7	0.78	23
Nov. 3	7	0.06	1

[a] Each value is the mean number of spores observed on 195 mm² of each of two slides

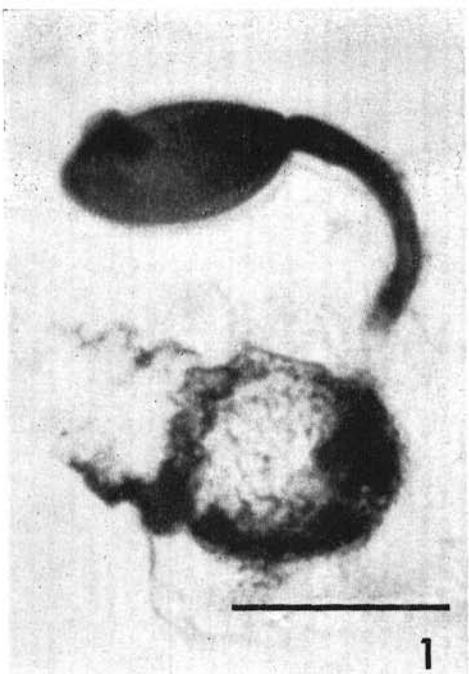
Plastic prints of Austrian, Scots, and ponderosa pine needle surfaces provided information on spore germination, germ tube growth, and the site of needle penetration. Spore germination and germ tube growth were more variable on needle surfaces than on water agar. The per cent germination of 75 spores and the mean length of 20 germ tubes were determined for each of eight pine needles incubated 4 hr at 26 C and 100% relative humidity. Thirty-four per cent of the spores germinated, and mean germ tube length was 117 μ. Eighty-eight per cent of spores from the same suspension germinated with a mean germ tube length of 214 μ after 4 hr on water agar at 26 C.

The fungus appeared to penetrate needles through stomata. Although some germ tubes grew directly toward and entered the nearest stomatal pit (Fig. 1.), while others grew past a stoma and entered another stomatal pit, most did not grow toward or enter stomata (Fig. 2). Some germ tubes grew directly over stomatal pits without entering. Similar results were obtained when fluorescence microscopy was used to observe spore germination and germ tube development on needle surfaces (Fig. 3). Per cent germination, germ tube length, and germ tube orientation did not appear to be affected by the species, age of needle on which the spores were incubated, or by fluorescent labeling.

Sections of needles inoculated with fluorescent-labeled spores provided additional evidence that stomata are the site of needle penetration. Over 10,000 needle sections were examined microscopically, and direct penetration of the epidermis was never observed. Fluorescent hyphal masses were commonly observed in stomatal pits (Fig. 4). These masses often extended above the stomatal pits because of the mass of hyphal growth near the bottom of the pits (Fig. 5). Hyphae from fluorescent hyphal masses entered stomata and grew into the mesophyll (Fig. 6). Although hyphae inside the mesophyll did not fluoresce brightly, they could definitely be traced to the fluorescent hyphal mass in the pit.

Infection and disease development.—Diplodia pinea caused rapid chlorosis and necrosis of young Austrian, Scots, and ponderosa pine needles when shoots were inoculated with spores and held at 100% relative humidity and 24, 26, or 28 C. Usually within 3 to 4 days after inoculation, small, tan to reddish-brown lesions appeared on the lower portion of several inoculated needles. These initial symptoms are easily overlooked, since a large portion of an infected needle often becomes chlorotic within 4 to 6 days after inoculation.

In nature, *D. pinea* infects young pine needles early in the growing season. The fungus frequently invades young stems, killing entire shoots. Small, black pycnidia are produced near the base of necrotic needles and on leaf-sheaths, stems, and cones. Pycnidia containing immature spores were observed as early as 13 June in microtome sections of young Scots pine shoots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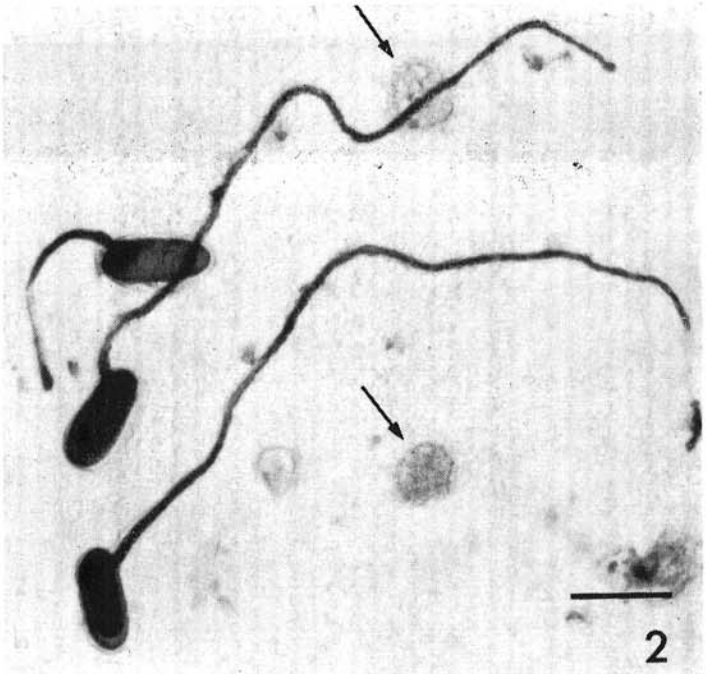
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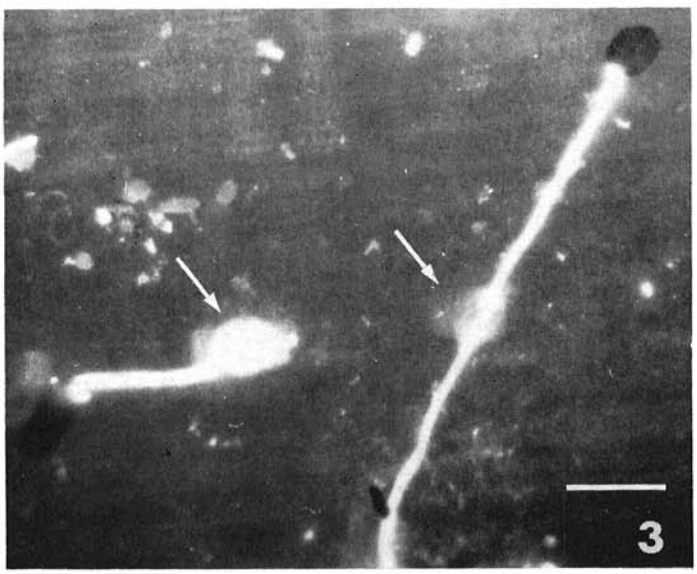
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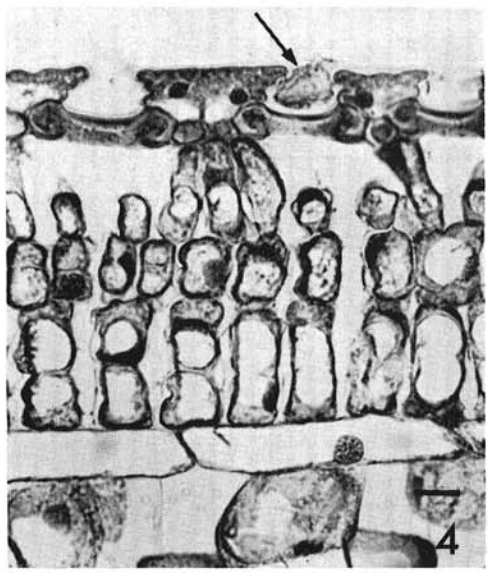
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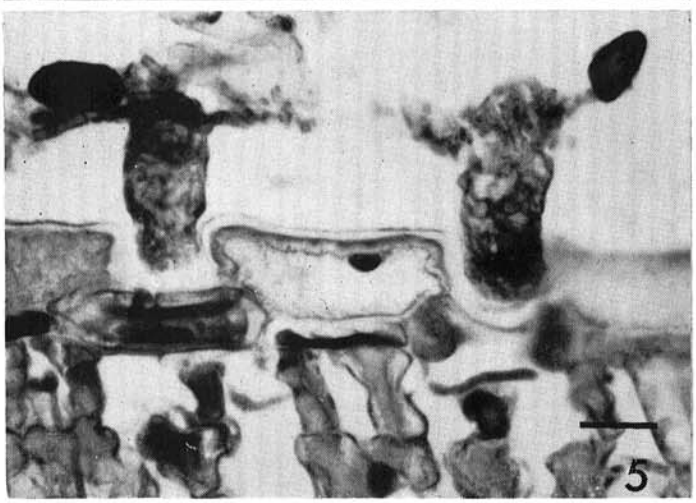
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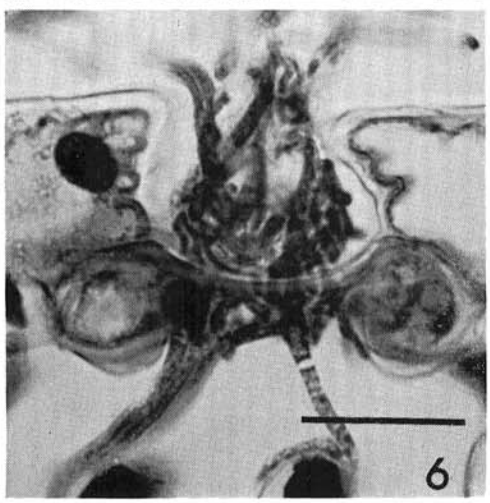
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TABLE 2. Infection of 10-year-old, field-grown Austrian, Scots, and ponderosa pine trees inoculated at various times with *Diplodia pinea* in 1969

Inoculation date	Austrian pine		Scots pine		Ponderosa pine	
	Needles[a] infected	Needle[b] length	Needles[a] infected	Needle[b] length	Needles[a] infected	Needle[b] length
	%	cm	%	cm	%	cm
April 28	96	0.0	100	0.0	100	0.0
May 5	100	0.1	100	0.2	100	0.0
12	100	0.1	100	0.2	91	0.0
19	82	0.6	36	0.5	96	0.4
26	92	0.9	77	0.8	100	0.9
June 9	70	1.1	42	1.3	75	2.0
23	11	2.0	38	1.5	23	3.7
July 8	5	2.9	6	2.1	16	4.1
24	1	3.1	6	2.3	1	4.6
Aug. 4	3	4.5	0	2.5	5	5.0
Sept. 2	1	4.3	1	2.6	0	5.2
16	0	4.6	4	2.5	0	5.3

[a] Four hundred and fifty needles (50 needles × 3 shoots × 3 trees) evaluated 19 October 1969. The early inoculations were made before needles emerged; a 100% infection rating was given each bud or shoot which died before needles emerged.
[b] Forty-five needles (5 needles × 3 shoots × 3 trees) measured at time of inoculation.

naturally infected with *D. pinea*. Pycnidia containing viable spores were found in early August on shoots infected that season.

Twelve hr at 100% relative humidity was sufficient for infection of uninjured pine shoots. Two-thirds of the Austrian and ponderosa pine shoots and all of the Scots pine shoots became infected when held at 100% relative humidity for 12 hr after inoculation. All shoots on Austrian, Scots, and ponderosa pines held at 100% relative humidity for 24, 36, or 48 hr became infected. Symptoms developed more rapidly on Austrian and Scots pine than on ponderosa pine. The pathogen was consistently reisolated from shoots expressing symptoms.

Inoculations in the field revealed that pine needles were most susceptible to infection from late April until mid-June; late summer inoculations resulted in very little infection (Table 2). The previous season's needles never developed disease symptoms. Pine buds inoculated 28 April 1969 became infected and failed to open. Symptoms developed more slowly on shoots inoculated in the field than on shoots inoculated in the greenhouse. In the field, needles usually did not become chlorotic until 2 to 3 weeks after inoculation, while plants inoculated in the greenhouse became chlorotic within 4 to 6 days.

Growth and Sporulation in culture.—Diplodia pinea isolates grew on potato-dextrose agar at temperatures from 8 to 36 C; growth was maximum at 28 C. Of the various media tested, potato-dextrose agar, malt extract agar, and pine needle extract agar were best for rapid growth. On these media, colony diameter of the fungus increased about 25 mm/day.

Pycnidia were not produced in the dark on any of the media tested. Pycnidia containing viable spores were produced in all the media tested when cultures were incubated at 28 C under 15-w fluorescent bulbs. Pycnidia usually formed on the bottom of the culture. Ten-day-old cultures on pine needle extract agar contained 30 pycnidia/cm^2. About one-third of these pycnidia had produced viable spores.

DISCUSSION.—Microscopic observations of *D. pinea* germ tubes entering stomatal pits and growing through stomata on uninjured pine needles established that wounding is not required for needle penetration. Subsequent infection and symptom development on these needles confirms further that wounding is not required for infection.

Although the fungus entered needles through stomata, growth of most germ tubes did not appear to be directed toward stomata. This contrasts sharply with another pathogen of pines, *Dothistroma pini*. Peterson (7) reported that more than 80% of *D. pini* germ tubes on Austrian and ponderosa pine needles grow toward stomata.

Diplodia pinea grows rapidly over a wide range of temperatures and on a variety of media. The optimum temperature for growth and the growth rate reported here are similar to those reported for other isolates of the fungus (2, 9, 12, 16). The optimum temperature and time required for germination are also similar to previously published results (12). The rapid germi-

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Fig. 1-6. **1)** Plastic print of a ponderosa pine needle surface showing a *Diplodia pinea* germ tube growing toward the nearest stoma. The marker in this and subsequent figures represents 25 μ. **2)** Plastic print of an Austrian pine needle surface made 4 hr after inoculation; germ tube growth is not directed toward stomata (arrows). **3)** A fluorescent-labeled germ tube growing into the nearest stomatal pit (arrow) and another growing directly over a stomatal pit (arrow) without entering. **4)** Mass of *Diplodia pinea* hyphae (arrow) in stomatal pit of an Austrian pine needle 5 days after inoculation. **5)** *Diplodia pinea* spores and hyphal masses above stomata of an Austrian pine needle 3 days after inoculation. **6)** *Diplodia pinea* hyphae growing from a stomatal pit, through a stoma, and into the mesophyll of an Austrian pine needle 3 days after inoculation.

nation and growth of the pathogen is in accord with the short time required for penetration, infection, and symptom development.

The results indicate that protection by fungicides is needed from late April until mid-June, because inoculum is abundant and pine shoots are highly susceptible during this period. Large numbers of spores are released only during periods of rainfall. Attempts to control *D. pinea* by delaying fungicide applications until rain falls probably would not be successful, however, since 12 hr at high humidity may be sufficient for infection of young shoots.

Peterson & Wysong (8) reported that damage to pines in Nebraska by *D. pinea* becomes severe on trees over 30 years old. They suggested that younger trees are not severely damaged due to the absence of pine cones, which produce abundant pycnidia. The results presented here indicate that shoots on 10-year-old Austrian, Scots, and ponderosa pines were highly susceptible to infection; thus lack of severe damage by *D. pinea* to young pine plantings in the central Great Plains is probably due to low inoculum levels within the plantings.

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