Development of Dothistroma pini Upon and Within Needles of Austrian and Ponderosa Pines in Eastern Nebraska

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ABSTRACT

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The growth of germ tubes of *Dothistroma pini* conidia was postively directed toward stomata on Austrian and ponderosa pine needles naturally inoculated in the field; however, tubes were seldom directed toward stomata on needles artificially inoculated and incubated at 100% relative humidity (RH). Germ tubes grew in a straight line when conidia were incubated on water agar in horizontally-or vertically-oriented plates. Conidia that had been hydrated for 8 hr, then dehydrated for up to 24 hr at temperatures up to 35 C, still were capable of germinating in vitro. Germ tubes of conidia incubated on inoculated needles for 24 hr at 24 C and 100% relative humidity (RH) and desiccated for 4 hr at 24 C and 30% RH, did not develop further even under conditions optimum for development. Stomatal density at needle tips was greater than at needle bases, but not sufficiently different to account for the higher rate of infection of needle tips. Penetration and development of *D. pini* were similar in both ponderosa and Austrian pine needles, but development was more rapid in the latter. Considerable development of the fungus occurred in stomatal pits before needles were pentrated. Penetrating hyphae emerged from disks that developed at the base of appressoria in stomatal pits. Hyphae were abundant in necrotic zones in the mesophyll, but discolored cells in adjacent zones were free of hyphae. Fruiting body development began in the necrotic mesophyll tissue. After symptoms had developed, hyphae were found in resin canals, in endodermal and transfusion tissues, and in parenchyma cells within vascular bundles.

Additional key words: *Pinus nigra*, *Pinus ponderosa*, *Scirrhia pini*.

Investigations of Dothistroma needle blight on ponderosa (*Pinus ponderosa* Laws.) and Austrian (*P. nigra* Arnold) pines in eastern Nebraska have led to the development of effective and economical controls for this disease in Christmas tree, shelterbelt, park, landscape, and other plantings in the Great Plains and elsewhere in the United States (10, 11, 13, 14, 15). This article presents results of current investigations on the development of *Dothistroma pini* Hulbary on and within needles of ponderosa and Austrian pines. This information was needed to understand infection and disease development.

In previous epidemiological studies, germination of *D. pini* conidia on Austrian and ponderosa pines was examined with plastic prints of needle surfaces (11). A high percent of germ tubes was postively directed toward stomata (12); however, other investigators (5, 8) have reported that germ tubes of *D. pini* conidia grew randomly on needles. In this article we present reasons for these differences and explore the possible cause for the directed growth.

Conidia dispersed on needles do not germinate when drying weather follows dispersal (13). Thus, tests were conducted to determine the extent of drying that previously hydrated conidia could tolerate and still germinate. This information is pertinent to the timing of fungicide applications following rain.

We attempted to determine if reported differences in optimum temperature for germination (7, 18) might be related to differences in germination media. This was deemed necessary since degree of infection in other areas has been explained on the basis of low optimum temperature for germination.

The amount of infection, as determined by numbers of lesions, has been reported to be greater near tips of needles than near the base (11). Since *D. pini* enters needles through stomata, counts were made to determine if there were differences in stomatal density between needle tips and bases that might account in part for the differences in lesion number.

Infection studies showed that, in some instances, long periods (more than 360 hr) of high humidity following inoculation were necessary for infection to occur (5, 9), but in other instances (3, 4), infection followed by production of *D. pini* conidia occurred within 2 wk of inoculation. To understand these differences, histological techniques were used to follow the development of the fungus within the host over a period which encompassed the life cycle of *D. pini* on Austrian and ponderosa pines in plantings in eastern Nebraska.

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MATERIALS AND METHODS

Direction of growth of germ tubes of Dothistroma pini conidia on pine needles.—To evaluate germ tube growth on pine needles in the field, needles were collected from 30- to 35-yr-old Austrian and ponderosa pines in eastern Nebraska. Collections were made 1 or 2 days after rain that resulted in dispersal of *D. pini* conidia. Plastic prints of needle surfaces which clearly showed germinating conidia and location of stomata were made with a solution of 4% cellulose acetate in acetone. Needles were coated by either spraying or dipping the needles in the solution. Films were stripped from needles, mounted in water, stained with aniline blue, and examined with a microscope. Germinating conidia were observed on 21 Austrian and eight ponderosa pine needle collections made over a 2-yr period.

In addition, *D. pini* conidia were germinated on needles in controlled-environment chambers to determine if growth of germ tubes toward stomata is due to a water gradient outward from stomata. Inoculations were made on: (i) needles attached to trees and (ii) detached needles whose bases were immersed in water in test tubes.

Inoculated needles were incubated at 24 C, 100% relative humidity (RH), and 16 hr/day light (approximately 12,900 lux) in ISCO environmental chambers. Plastic prints were made of the surfaces of three attached and three detached current-year needles for each incubation time (24, 36, and 48 hr). Four-yr-old trees were used in one test and 2-yr-old trees in a second test. Data were obtained on: (i) percentage of germinated conidia, (ii) number of germ tubes on each germinating conidium, (iii) length of germ tubes, (iv) number of tubes growing toward stomata, (v) number of tubes growing in a curved path toward stomata, and (vi) distance tubes grew before turning toward stomata.

To determine if there is a geotropic growth response by germ tubes, a suspension of conidia was placed on each of six petri dishes which then were incubated either in a horizontal or vertical position at 24 C for 24 or 36 hr.

Influence of drying on germination of Dothistroma pini conidia.—Conidia were hydrated either by immersing conidia in water or by placing conidia on dialysis membranes on water agar. After hydration, the conidia were exposed to drying conditions (low RH) for various periods at various temperatures. Conidia then were incubated on water agar (24 C) for 24 hr, and percent germination was determined. In the first test, needles with fruiting bodies were incubated for 40 hr at 100% RH; then fruiting bodies were removed and crushed in a watch glass to release conidia. Conidia were immersed in water for various periods, then dried for various periods on dialysis membrane sections. In the second test, needles with fruiting bodies were incubated in dew chambers at 100% RH for 62 hr, and the conidia were obtained as before. These conidia were placed on dialysis membranes laid on water agar for various periods to allow hydration before drying (20% RH) for various periods and at various temperatures. In both tests, the dialysis membranes were returned to water agar plates for germination counts. In a third test, inoculated needles on Austrian and ponderosa pine seedlings were incubated for 24 hr (24 C, 100% RH), placed in a drying atmosphere (24 C, 30% RH) for 4 hr, and then incubated for an additional 24 hr (24 C and 100% RH). Plastic prints of needle surfaces were used to obtain germination data.

Germination of Dothistroma pini conidia in water and on water agar.—Conidia were obtained from fruiting bodies on Austrian pine needles which had been kept at 24 C and 100% (RH) for 10 hr. Conidia were placed with a glass rod on water agar in plates and on cover slips held on microscope slides by petrolatum. Plates and slides were incubated at 16, 20, 22, 24, and 28 C. Slides were kept at 100% RH. At specific time intervals, conidia and germ tubes were killed with 0.1% $HgCl_2$. Percent germination was determined from 100 conidia, and lengths of 30 germ tubes were measured in each of three replicates. If conidia had more than one germ tube, only the longest tube was measured.

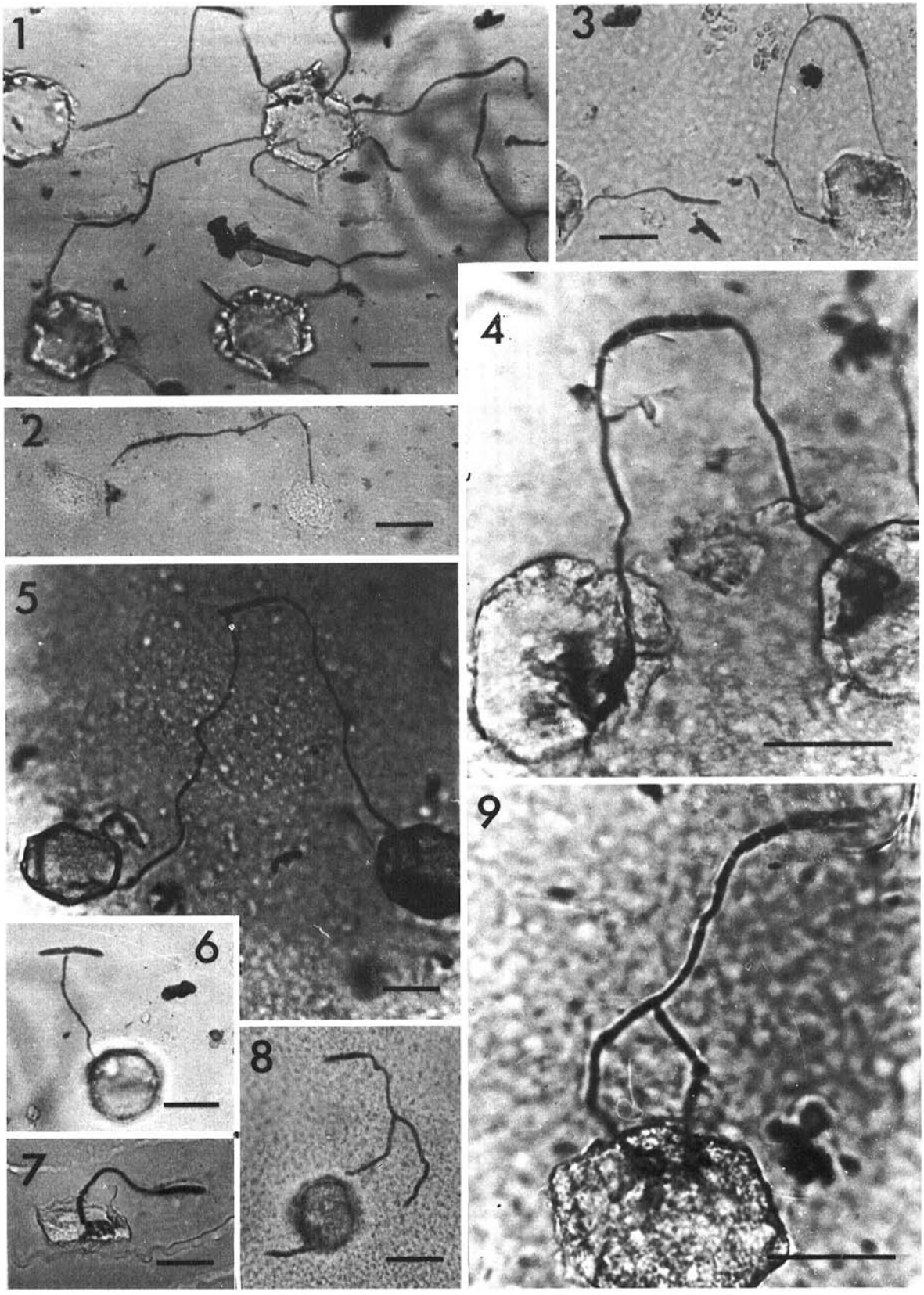
Stomatal density on pine needles.—Counts of stomata were made on Austrian and ponderosa pine needles collected in late July 1965 in Pioneers Park, Lincoln, NE. Needle sections (3.5 mm long) were cut 25 mm and 64 mm from the top and 13 mm from the base. Stomata were counted on abaxial (curved) and adaxial (flat) needle surfaces. Adaxial needle surfaces were determined with the epidermis in place, but for abaxial surfaces the epidermis was stripped off and flattened.

Several needle collections were made for the purpose of comparing stomatal density (i) on needles of the same tree collected from shoots with either high or low exposure to light, (ii) at different intervals along the length of needles, (iii) on different surfaces of needles (abaxial vs. adaxial), (iv) on different-aged needles from the same tree, and (v) on needles from trees either resistant or susceptible to *D. pini*.

Development of Dothistroma pini within pine needles.—Austrian and ponderosa pine needles which developed in 1965 were collected weekly from 1 July 1965 through 26 November 1966 from 30 to 35-yr-old trees in eastern Nebraska. In 1965, 95% of the Austrian pine needles and 54% of the ponderosa pine needles were infected (11). Segments 5 mm long were cut 10 to 15 mm from needle tips of symptomless needles; from needles with symptoms, 5-mm segments were cut which encompassed the most advanced symptoms. These segments were kept in sterile distilled water from 2 to 4 hr, then fixed in formalin-acetic acid-alcohol (FAA). The segments were dehydrated with butanol (16) and embedded with paraffin (Parawax-48 C) with an Autotechnicon. Segments were softened by trimming the paraffin blocks and placing them in a solution containing one part glacial acetic acid and four parts 60% ethanol (6) for 2 to 5 days. Blocks containing the softened segments were placed in distilled water one day before cutting. Longitudinal sections 14 μm thick were cut in series with a rotary microtome and stained with Pianeze III-B (19).

RESULTS

Germination of Dothistroma pini conidia on needles of Austrian and ponderosa pines.—The direction of growth of *D. pini* germ tubes on needles collected from the field was positively directed toward stomata (Fig. 1). Tubes often abruptly changed growth direction toward stomata (Fig. 2). Tubes from several conidia were observed in the same stoma (Fig. 1). Two tubes from a conidium often grew in a curved path toward the same stoma (Fig. 3) or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occasionally into different stomatal pits (Fig. 4, 5). Sometimes germ tubes developed from the side of a conidium nearest a stoma and grew directly into the stomatal pit (Fig. 6). The tubes branched and developed within the stomatal pits (Fig. 7). Branches of a single germ tube often grew in a curved pattern toward a stoma (Fig. 8, 9). Germ tubes grew toward stomatal pits of resistant as well as susceptible needles. There was no indication that needle topography, such as ridges or depressions, influenced germ tube orientation.

Secondary conidia were produced on needles from one collection made after several days of highly moist coniditions. Conidia were produced on germ tubes; these multiseptate conidia then produced conidia from each of their cells.

On artificially inoculated needles at 100% RH only 7% to 13% of the germ tubes grew in a curved pattern toward stomata. The distance tubes grew before curving toward stomata averaged 29 to 34 μm.

Germ tubes grew in a straight line on water agar. Orientation of petri dishes had no effect on direction of growth or length of germ tubes.

Influence of drying on germination of Dothistroma pini conidia.—Desiccation of hydrated conidia on dialysis membranes did not prevent germination, although percent germination was reduced with increased drying (Table 1, 2). However, germ tubes of conidia on inoculated ponderosa and Austrian pine needles which had been incubated for 24 hr at 24 C and 100% RH did not develop further after desiccation for 4 hr at 24 C and 30% RH, even when placed at optimum conditions for 24 hr.

Germination of Dothistroma pini conidia in water and on water agar.—Germination and germ tube growth were optimum at 24 C on either water agar or in water (Fig. 10). Germination was near optimum at 22 C, but decreased rapidly below 22 C or above 24 C. Percent germination and germ tube lengths were greater on water agar than in water. Conidia germinated 2 to 4 hr earlier, and germ tubes grew faster on water agar than in water. Some germ tubes were present within 8 hr on conidia incubated at optimum temperature (24 C), but no tubes were observed within 6 hr on either water agar or in water.

Stomatal density on pine needles.—Stomatal density was less at 13 mm from the base of needles than at 25 or 64 mm from the tip of needles, particularly in second-year needles. Stomatal densities 25 and 65 mm from needle tips were similar. There were no marked differences in stomatal density between needles resistant or susceptible to *D. pini*. On the average, there were slightly more

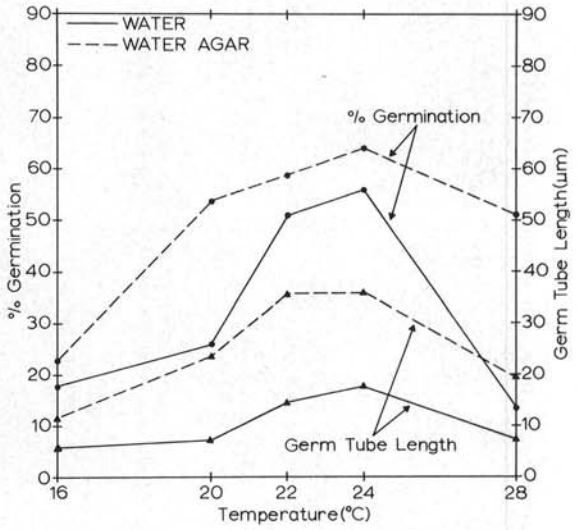

Fig. 10. Germination of *Dothistroma pini* conidia after 24 hr on water agar or in water at various temperatures.

TABLE 1. Germination of *Dothistroma pini* conidia which were hydrated then desiccated at 25 C and 23% relative humidity for various periods

Drying time (hr)	Germination after hydration for: 0 hr (%)	3 hr (%)	6 hr (%)
0	56	55	47
1	61	52	49
2	57	38	51
4	54	51	51
6	56	42	46
8	60	48	48
24	44	42	40

[a]Basis: 600 conidia (three replications × 200 conidia). Conidia were incubated on water agar (24 C) for 24 hr.

TABLE 2. Germination of *Dothistroma pini* conidia following hydration on water agar and then desiccation at 20% relative humidity for various periods

Hydration time (hr)[a]	Drying time (hr)	Germination[b] after drying at: 24 C (%)	29 C (%)	35 C (%)
0	0	49	...	...
0	8	48	41	37
0	24	36	33	25
8[c]	24	35	33	43

[a]Conidia placed on membrane sections on water agar at 24 C for presoaking.

[b]Basis: 600 conidia (three replications × 200 conidia). Conidia incubated on water agar (24 C) for 24 hr.

[c]Germination of 1.5% occurred after 8 hr of presoaking.

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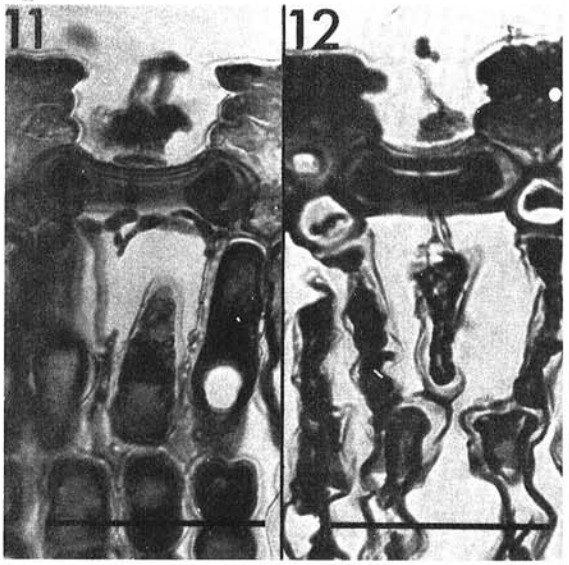
Fig. 1-9. Plastic prints of Austrian pine needle surfaces showing *Dothistroma pini* conidia and germ tubes and impressions of stomata. **1)** Many germ tubes growing towards and into stomatal pits. **2)** Germ tube which abruptly changed direction toward a stomatal pit. **3)** Two germ tubes of a conidium growing towards one stomatal pit. **4, 5)** Two germ tubes from a single conidium, each growing into a different stomatal pit. **6)** Germ tube from a central cell of a conidium growing toward a stomatal pit. **7)** Branching and development of a germ tube within a stomatal pit. **8)** Two branches from a germ tube of a conidium, both growing toward a single stomatal pit. **9)** Two branches from a germ tube of a conidium, both growing into a single stomatal pit. Bar = 10 μm.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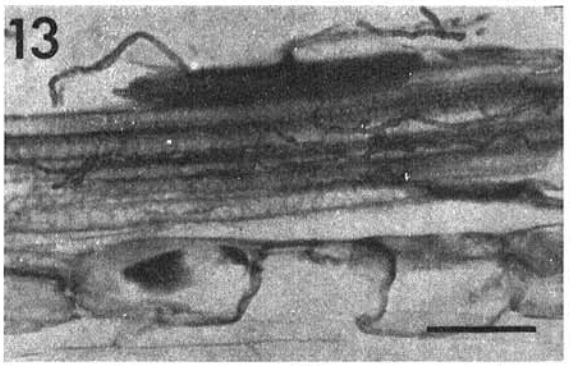
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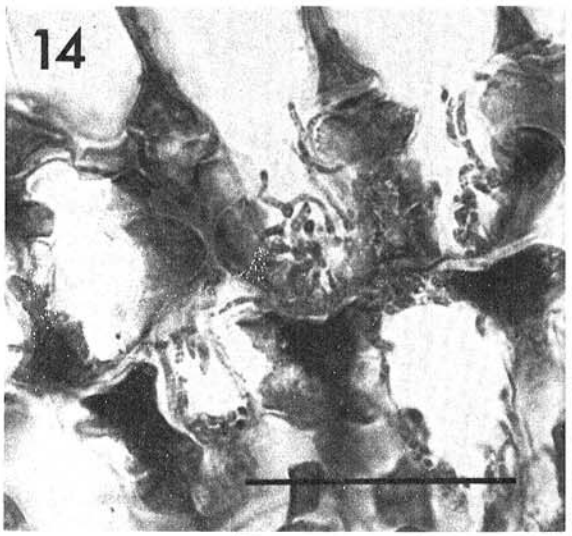
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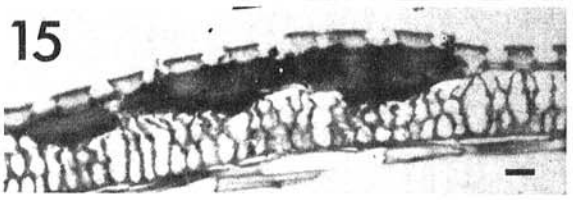
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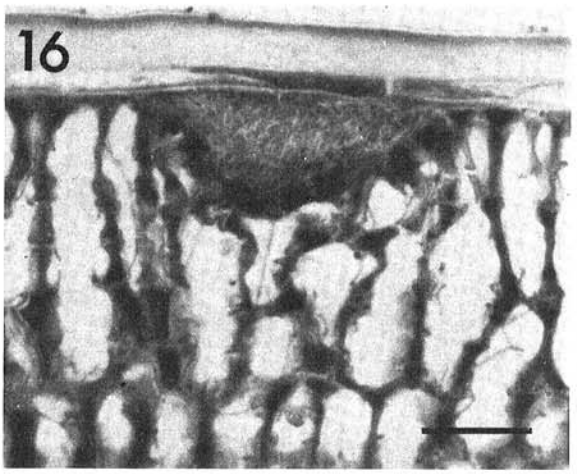
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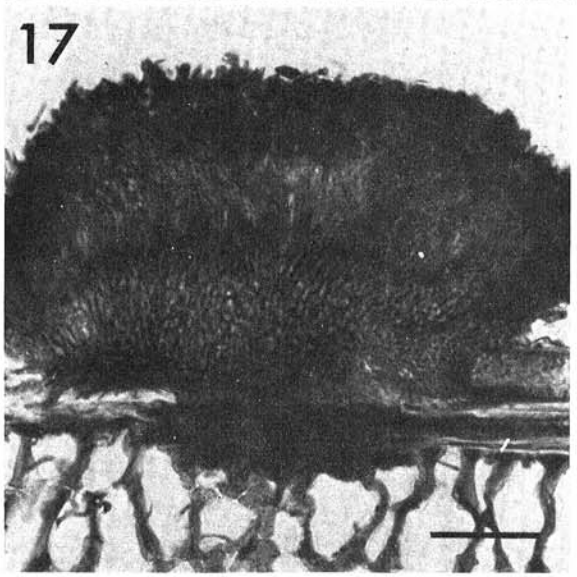
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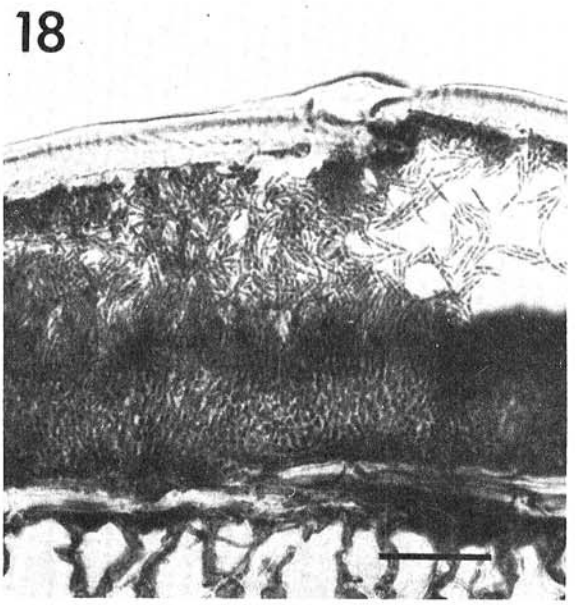
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stomata on needles of Austrian and ponderosa pines exposed to direct sunlight than on needles not exposed to direct sunlight (Table 3). Stomatal density was slightly higher on adaxial surfaces than on abaxial surfaces of needles of both species.

Development of Dothistroma pini within needles of Austrian pine.—Beginning 1 July 1965, nearly every needle throughout the collection period had appressoria which developed in stomatal pits. Beginning 29 July 1965, needles from all collection dates had hyphae entering through stomata. Within a 5-mm segment of a row of stomata, penetrating hyphae were observed in as many as nine stomata. Penetrating hyphae emerged from a disk that developed along the bottom of the appressorium (Fig. 11, 12). The bottom of the disk was smooth and curved, fitting the bottom of the stomatal pit.

In early infection stages, intercellular hyphae were found in healthy appearing mesophyll (Fig. 11). No substomatal vesicles were observed. Hyphae often grew a considerable distance toward the endodermis before they branched and spread throughout the mesophyll. Before more than three hyphal strands developed, cells within the mesophyll collapsed (Fig. 12). This caused necrotic zones which were first noted in needles collected 19 August, 1965. Zones between the collapsed and healthy tissues were free of hyphae and stained various colors by Pianeze III-B.

Most hyphae were in the mesophyll; however, hyphae were observed in resin canals, endodermis, transfusion, and vascular tissues. Mycelium was more common in resin canals than in endodermal, transfusion, and vascular tissues. Hyphae were first observed in resin canals of needles collected 22 October 1965, in the endodermis of needles collected 13 November 1965, and in transfusion tissue of needles collected 26 November 1965. Both inter- and intra-cellular hyphae were observed in transfusion tissue. Hyphae were first found in parenchyma cells interspersed within a vascular bundle

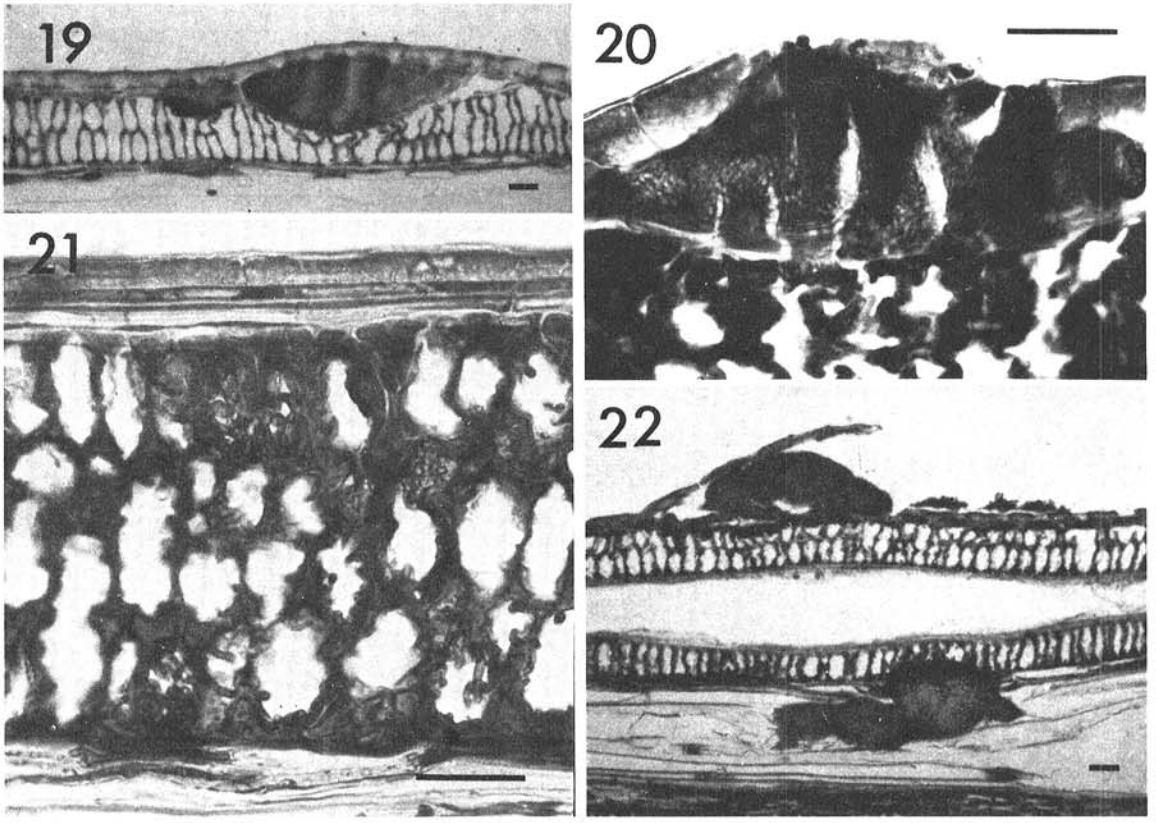

Fig. 11-22. Longitudinal sections of 1965 Austrian (AP) and ponderosa (PP) pine needles infected by *Dothistroma pini*. **11)** Appressorium showing disk with budded hyphae penetrating between healthy tissue in an AP needle collected 5 August 1965. **12)** Appressorium showing disk with hyphae penetrating collapsed tissue in an AP needle collected 26 August 1965. **13)** Hyphae in parenchyma cells interspersed within the vascular bundle in an AP needle collected 3 June 1966. **14)** Hyphae in intercellular spaces of collapsed mesophyll in AP needle collected 23 December 1965. **15)** Large fruiting body becoming erumpent on an AP needle collected 11 December 1965. **16)** Small fruiting body in mesophyll of an AP needle collected 23 December 1965. **17)** Prosenchymatous and pseudoparenchymatous tissues within a fruiting body on an AP needle collected 27 May 1966. **18)** Sporiferous fruiting body on AP needle collected 27 May 1966. **19)** Columnated fruiting body on AP needle collected 23 February 1966. **20)** Columnated fruiting body on AP needle collected 13 May 1966. **21)** Mass of hyphae in pine needle collected 29 April 1966. **22)** Fruiting body within the vascular tissue of a PP needle collected 3 June 1966. Bar = 50 μm.

on needles collected 13 May 1966 (Fig. 13).

The first *D. pini* fruiting bodies observed were on needles collected 7 October 1965. Groups of fruiting bodies were common in needles collected 4 December 1965 and later. Fruiting bodies usually were centered in necrotic zones in the mesophyll. The associated masses of mycelium varied in density, at times consisting of hyphae packed and intertwined (Fig. 14).

The fruiting bodies varied from an erumpent, very large mass along the surface of the needles (Fig. 15) to a small mass of mycelium along the inner edge of the hypodermis (Fig. 16). The more-developed fruiting bodies contained prosenchymatous and pseudoparenchymatous cells (Fig. 17).

As the prosenchymatous cells developed, they occupied an increasing portion of the fruiting bodies. Differential structure in some fruiting bodies indicated early stages of conidial development (Fig. 17). Conidia were located in the area which contained prosenchymatous cells in less-well-developed fruiting bodies (Fig. 18). A thin layer of pseudoparenchymatous cells covered the prosenchymatous cells or the immature conidia (Fig. 17) if they were not covered by epidermal cells. Conidia often developed along the entire length of fruiting bodies, but sometimes developed only along the perimeter where fruiting bodies were exposed to air due to a ruptured epidermis. Conidia were first observed within fruiting bodies on 1965 needles collected 13 May 1966. The perfect stage (*Scirrhia pini* Funk & Parker) of *D. pini* (1) was not observed.

Columnated fruiting bodies were observed in needles collected 23 February 1966 and later. The columnation occurred in two forms: one as separation into alternate light and dark areas within the fruiting body (Fig. 19) and the other as complete separation of portions of the fruiting body (Fig. 20).

Dothistroma pini development within needles of ponderosa pine.—Appressoria development and penetration of stomata in ponderosa pine needles were similar to those in Austrian pine needles. Appressoria were observed in needles collected 22 July 1965 (1 July for Austrian) and on all subsequent dates. Hyphae first were observed entering stomata on needles collected 16 September 1965 (29 July for Austrian). The number of penetrating hyphae was much lower in ponderosa than in Austrian pine.

Collapsed mesophyll was first observed in needles collected 30 September 1965. The necrotic zones were smaller overall in ponderosa pine than in Austrian pine needles. Furthermore, the zone between healthy and collapsed tissues did not have a colored intercellular material as in Austrian pine needles.

There were dense masses of mycelium in ponderosa pine needles (Fig. 21), but generally there was less mycelium in ponderosa than in Austrian pine needles.

Fruiting bodies were first observed 22 October 1965 in ponderosa needles. Fruiting bodies on needles collected 3 June 1966 contained prosenchymatous and pseudoparenchymatous cells, which indicated an early stage of sporulation. Both mycelium and a fruiting body were observed in one instance in the endodermis, transfusion tissue, and vascular tissue of a needle collected 3 June 1966 (Fig. 22).

In general, *D. pini* development in ponderosa pine was similar but slower than in Austrian pine.

TABLE 3. Stomatal density at different intervals along adaxial and abaxial surfaces of first- and second-year Austrian and ponderosa pine needles from sunny and shady locations

Location on needles[a]	Stomatal density[b]			
	Adaxial surface		Abaxial surface	
	Sun (no./mm^2)	Shade (no./mm^2)	Sun (no./mm^2)	Shade (no./mm^2)
Austrian pine				
First-year needles				
25 mm from tip	56	55	48	47
64 mm from tip	42	46	44	45
13 mm from base	45	33	40	43
Second-year needles				
25 mm from tip	63	58	54	49
64 mm from tip	56	49	51	45
13 mm from base	29	18	45	35
Ponderosa pine				
First-year needles				
25 mm from tip	64	59	62	56
64 mm from tip	65	54	65	56
13 mm from base	53	43	57	49
Second-year needles				
25 mm from tip	71	61	62	56
64 mm from tip	69	60	65	53
13 mm from base	39	32	43	39

[a]Needle sections were 3.5 mm in length.
[b]Each datum is an average from five needles from the same shoot.

DISCUSSION

The chances that needles will be infected by *D. pini* are increased when germ tubes grow toward stomata as opposed to germ tubes that grow at random. Investigators in New Zealand (2) and in East Africa (8) have reported that germ tubes of *D. pini* were not positively directed toward stomata on Monterey pine (*Pinus radiata* D. Don) needles. However, their observations were made of artificially inoculated needles incubated at 100% RH. With artificially inoculated Austrian and ponderosa pines incubated at 100% RH, we found that germ tubes seldom were directed toward stomata. It was only when we examined surfaces of needles naturally inoculated in the field that high numbers of positively directed germ tubes were observed. We suggest that positive direction of germ tube growth toward stomata is a more common phenomenon than has been indicated in the literature.

In this regard, the direction of conidial germ tube growth of another needle pathogen, *Scirrhia acicola* (Dearn.) Siggers, has been reported as random on Scots pine (*Pinus sylvestris* L.) needles (17). The observations were made on needles of artificially inoculated seedlings incubated under high relative humidity. However, our observations (*unpublished*) of naturally inoculated Scots pine needles from trees growing in the field revealed that most (72%) germ tubes of *S. acicola* conidia were positively directed toward stomata.

Our observations of *D. pini* did not reveal that direction of germ tube growth was related to needle topography, but a water gradient from spores to stomata may be involved.

Secondary conidia were observed on surfaces of needles collected in the field, but only following a lengthy period of high moisture. Production of secondary conidia on artificially inoculated needles incubated at high RH was reported by Gadgil (2).

Hydrated conidia were still capable of germinating after relatively long periods of dehydration in vitro; however, only 4 hr of desiccation of germinated conidia on needles was sufficient to stop further germ tube development. This suggests that a brief drying period, occurring after dispersal of conidia by rain splash in the field and after germination had commenced, would result in little or no infection even if conditions favorable for germ tube development were present subsequently. These findings are consistent with Gadgil's (4) results on infection of inoculated Monterey pine seedlings exposed to different wet and dry periods.

The optimum temperature (24 C) for spore germination and germ tube development in our tests was higher than that obtained by investigators in New Zealand (18) and in East Africa (7). Although the stomatal density of needle bases was lower than that of needle tips, the magnitude of the difference is not considered large enough to account for the lower number of lesions on bases than on tips of current-year needles as reported by Peterson (11).

Penetration of stomata did not occur until there was considerable growth and development of the fungus within the stomatal pit, which were dependent on very moist conditions. Parker (9) did not obtain infection of ponderosa pine seedlings unless the seedlings were exposed to 550 hr of 100% RH. Similarly, Garcia (5) did not get infection of Monterey pine seedlings unless the seedlings had been exposed to 15 days of high humidity.

Prior to penetration, *D. pini* is apparently vulnerable to fungicides such as Bordeaux mixture. Tests on time of infection conducted in 1965 by Peterson (11) revealed that no infection of current-year needles occurred on needles that had received one fungicide application on 1 July; however, our histological study revealed that there already was considerable development of *D. pini* in stomatal pits by 1 July, but that penetration occurred later in the month.

The time of growth of the fungus through the stoma and into the mesophyll does not coincide with the time of symptom development on Austrian pine in eastern Nebraska. External symptoms were first evident 16 September 1965, but some mesophyll cells in Austrian pine needles had collapsed 1 mo before this.

Dothistroma pini can cause symptoms in a short period of time from inoculation (2 wk or less in artificially inoculated needles) (3), but both in Nebraska and in New Zealand the period from time of natural inoculation to development of symptoms may be as long as 11 wk. Our histological observations did not reveal any reason for variation in time of symptom development, but previous work had suggested that low temperatures may be involved.

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