Conidial Stage of Hypoxylon pruinatum: Factors Influencing Pillar Production and Conidial Germination

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ABSTRACT

Germinating conidia of *Hypoxylon pruinatum* occasionally developed into colonies on agar culture media. These colonies were always the "conidial"-type in contrast to the normal mycelial wild-type. Contact between colonies of different paired mycelial isolates was required for conidial development at merging colony margins. Conidia were always uninucleate. The optimum temp for conidial pillar development on naturally infected stem pieces of *Populus tremuloides* was between 20 and 24 C. Relative humidities lower than 95% resulted in inhibition of conidial pillar development. Mycelial macerates of certain isolates of the fungus, when mixed aseptically in warm oatmeal agar prior to gelation, produced conidial pillars in pure culture.

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The conidia of *Hypoxylon pruinatum* (Klot.) Cke. are hyaline, unicellular, fusiform to elliptical, approximately 3×6 μ in size, and are borne on 75 to 150-μ long, compound, dichotomously branched, geniculate conidiophores which, in nature, cover most of the surface of $0.85 \pm 0.2 \times 1.5 \pm 0.2$ mm peg-like conidial pillars, or conidial stromata. These stromata are borne under the periderm on the developing canker of parasitized quaking aspens (*Populus tremuloides* Mich.). Conidia develop on these pegs in great abundance and appear as olive-green masses under the broken periderm (8).

The role conidia play in the natural spread of Hypoxylon canker is not known. Ascospores appear to constitute the major source of inoculum for spread of the fungus in nature (3). However, Bagga and Smalley (1) observed that wound inoculations with ascospores were only successful when combined with topical applications of phytotoxic culture filtrates of the pathogen. Berbee and Rogers (3) suggest that conidia are probably not involved in vegetative propagation of the fungus, but function as spermatia. This remains to be established, however, since perithecia of the fungus have never been successfully produced in culture.

Most investigators have been able to germinate the conidia on agar (3, 4, 6, 7, 8). Ponomareff (8) found that cultures from conidia grown on malt agar were morphologically similar to those obtained from ascospores, and that they exhibited a wide range of variation. Bier (4), however, observed that most germinated conidia collapsed after 5-7 days, although a few of them subsequently developed into colonies. Berbee and Rogers (3) reported that conidia never produced colonies.

Single ascospore isolates of *H. pruinatum* differed in morphological characters, growth rates, and the amount of conidial production (1, 4, 7). Bier (4) derived four distinct cultural types from the eight ascospores in single asci. When these isolates were paired in all possible combinations, the colonies grew together in only four of the 27 pairings. Other pairings produced a distinct line of demarcation between colonies.

The structure of the conidial pillar (conidial stroma) has been elucidated by Rogers and Berbee (9). Barnett (2) showed that *Hypoxylon punctulatum* (Berk. & Rav.) Cke. produces a *Basidiobotrys* conidial stage on pillars prior to stroma formation. Wehmeyer (12) reported the presence of similar bark-rupturing fungal structures of *Diatrype stigma* (Hoff.) de Not. Detailed investigations on the factors affecting the formation of conidial pillars of *H. pruinatum* have not been previously reported.

The present investigations include studies on factors influencing conidial germination in vivo and conidial pillar development in vitro.

MATERIALS AND METHODS.—Conidial suspensions for use in these studies were obtained from either naturally infected aspen cankers or from pure "conidial"-type cultures. The conidial suspension was shaken and centrifuged at 500 *g* for 10 min to remove contaminating hyphal fragments. The supernatant was then passed through a double layer of Reeve Angel No. 202 filter paper. Microscopic examination of these suspensions revealed only conidia. Conidial suspensions in viability tests were dispersed into petri plates

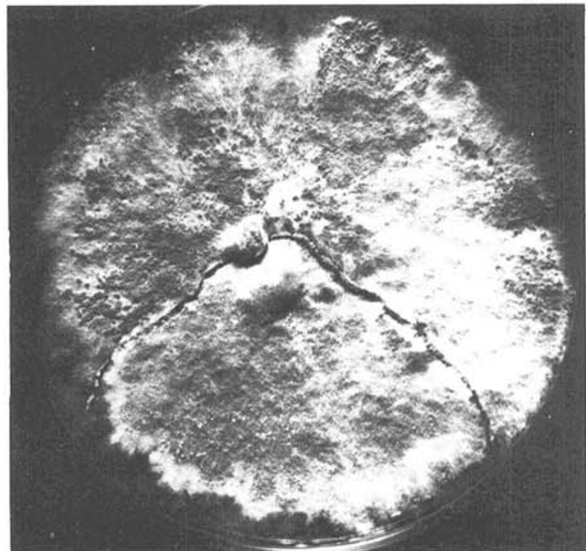

Fig. 1. Conidial streak developing 2 wk after pairing of two different single ascospore isolates of *Hypoxylon pruinatum* on oatmeal agar at 24 C.

TABLE 1. Development of conidial pillars on naturally infected aspen stem pieces at different temp after 14 days of moist chamber incubation

Temperature (C)	Conidial pillar development rating[a]
12	B
16	B
20	P
24	P
28	B + P
32	--[b]

[a]B = Blister stage; B + P = blister stage with some pillar stage; P = predominantly pillar stage.
[b]Results obscured by mold development.

containing 50 μg/ml streptomycin sulfate in potato-dextrose agar, malt agar, or V-8 juice agar (10). Similar trials were repeated on separate occasions. Cultural studies on conidial streak and conidial pillar development utilized malt agar or oatmeal agar as standard substrates.

Naturally infected aspen stem pieces (8-9 cm in diam × 10-18 cm long) with early-stage infections (pale green bark patches surrounded by 1- to 2-mm-wide yellowish margins) for studies on conidial pillar development were incubated in large, loosely capped, wide-mouth jars containing moist paper towels.

RESULTS.—*Conidial germination.*—Fewer than 10% of the conidia germinated in most of these trials. Occasionally, the germinating conidia developed into colonies, and such colonies were always of the conidial type in which the entire colony surface was covered with conidia. However, when individual, germinating conidia were transferred from the original plate to malt agar tube slants, no colonies were formed.

Conidial streak development.—Pure cultures of *H. pruinatum* from infected aspens always yielded "mycelial"-type cultures which rarely produced conidia, except occasionally in old cultures. When malt agar or oatmeal agar in petri plates was seeded with different paired mycelial isolates of *H. pruinatum,* growth of certain paris merged; in other pairings, the two colonies failed to merge, leaving uncolonized clear areas at the margins of the colonies. This clear zone was termed the "line of demarcation." In certain pairings, when the colonies were 2 wk old, a conidial streak developed at this line of demarcation (Fig. 1). In others, the line of demarcation remained without conidia. Conidia developed only where the two colonies met. In certain pairings, the conidial streak developed on only one side of the line. This relationship was not consistent, but the conidial streak in any particular pairing always developed towards one of the isolates. Dark-brown pigment in the medium was formed under the conidial streak. If a small fragment from the streak was transferred to malt agar, conidial cultures (compact, grayish, heavily sporulating colonies) developed instead of either parental type. Transfers away from the streak in the colony gave typical mycelial colonies. As the substrate dried, however, the parental cultures sometimes produced conidia. Cultures obtained from mass transfers of conidial streaks remained conidial.

To determine whether actual contact of mycelia of two cultures in the plate was necessary to induce the conidial streak, a piece of sterile collodion film was implanted in the center of a petri dish prior to pouring the melted agar. The collodion film projected slightly above the surface of the medium. Inocula from two isolates producing the conidial streak were paired with the membrane separating the two. Actual mycelial contact was apparently required, since no conidial streak was produced at the film barrier.

To obtain quantitative information on this effect, eight cultures derived from the eight ascospores (numbered 1 to 8 from the proximal end) from a single ascus (5) were paired in all possible combinations in petri dishes on malt agar. When cultures of isolates 1 and 2, 3 and 4, 5 and 6, 7 and 8 were paired, the colonies merged into each other without conidial production at the margin. Similar results were obtained when transfers of the same ascospore isolate were paired (i.e.; 1 × 1, 2 × 2, etc.). The remaining pairings between isolates from the ascus (i.e.; 1 × 3, 2 × 4, etc.) produced a line of demarcation and ultimately a conidial streak.

Since cultures obtained from conidia produced at the conidial streak always remained the conidial type, it suggested the possibility that conidia might be heterokaryotic. However, conidia stained with Giemsa stain (11) were found to be uniformly uninucleate.

The development of the conidial pillar.—After 4 days of incubation in the moist, wide-mouth jars at 24 C in the laboratory, the periderm over the green patches of the naturally infected aspen stem pieces became blistered. After 7 or 8 days, the periderm rose, split along the margins of the blisters, and conidial pillars under the periderm became visible. If the periderm at the blister stage was removed, the conidial pillars failed to develop.

To determine the reasons for the failure of conidial pillars to develop where periderm was removed at the blister stage, 12 similar naturally infected aspen stem pieces were incubated in the same way. When the periderm had loosened (3-4 days), it was removed and replaced with lightly waxed tissue paper, commercial wax paper, bond writing paper, or aluminum foil. All the covers were held tightly in position with tape. In the controls, the periderm either was left intact, or was removed at the blister stage.

The conidial pillars completed their development in treatments where blisters were left intact. Over half of the blocks in which the periderm was replaced with a sterile, lightly waxed tissue paper also produced pillars. The conidial pillars in all other treatments failed to complete their development.

When the periderm at blister stage was removed in the dark, and the material was incubated in dark for 10 days, conidial pillars in the exposed areas also failed to complete their development.

Effect of temp on conidial pillar development.—Using this detached technique, naturally infected stem pieces with early-stage infections were incubated at 12, 16, 20, 24, 28, and 32 C each with three replicates. The paper towels were kept moist by adding 15 ml of water every third day.

After 14 days at 12 and 16 C, the conidial pillars reached the blister stage, but failed to develop further. The optimum temp for development of conidial pillars

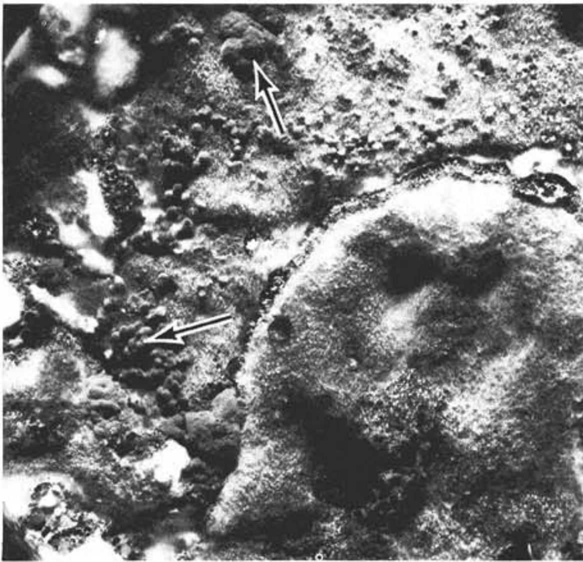

Fig. 2. Conidial pillars (arrows) produced in 30 days at 24 C on oatmeal agar following growth of isolates A and F of *Hypoxylon pruinatum* seeded as a mixed hyphal macerate in the warm ungelled agar.

was between 20 and 24 C. At these temp the blister stage appeared in 4 days; and in 10 days, pillar development was complete (Table 1). At 32 C, saprophytic fungi covered the experimental material, and pillar development could not be observed.

Effect of relative humidity (RH) on conidial pillar development.—Using the same technique, naturally infected stem pieces were incubated at 24 C in plant growth chambers at RH of 50%, 75%, 95%, and 100%. The conidial pillars only completed their development at 100% RH, although development progressed to the blister stage at 95%.

Production of conidial pillars in culture.—The ease with which conidial pillars developed from detached cankered aspen branches in moist chambers suggested that they might also form in pure cultures under appropriate conditions. Thirty conidial type colonies, half on malt agar and half on oatmeal agar, were grown for 2 wk. Five colonies on each medium were then covered with 4-cm disks of either propylene oxide-sterilized commercial waxed paper, lightly waxed tissue paper, or ordinary bond writing paper. The plates were then incubated in the dark at 24 C. After 4 wk, the colonies were covered with conidia, but conidial pillars failed to develop.

In another trial, two single ascospore isolates (which were known to produce conidial streaks when paired) were aseptically macerated separately in 10 ml of sterile distilled water and then mixed. The mixture was then distributed into five sterilized petri plates and covered with 25 ml of warm oatmeal agar and incubated at 24 C. After 30 days, conidial pillars scattered in small groups over the surface of the colony appeared in two of the five plates (Fig. 2).

DISCUSSION.—When germinating conidia of *H. pruinatum* occasionally developed into colonies, these colonies were always of the conidial types. These results were contrary to those reported by Bier (4), who obtained both mycelial and conidial colonies. Berbee and Rogers (3), however, suggested that the conidia functioned as spermatia. Bagga and Smalley (1) found that aspens became infected when conidial suspensions were injected into leaf axils, suggesting that conidia may be of some importance in natural pathogenesis.

Development of conidial streaks in single ascospore derived mycelial cultures required actual colony contact with different paired lines. Conidia derived from these pairings were always uninucleate.

Conidial pillar development was achieved in the laboratory on detached, naturally infected aspen branches incubated in moist chambers. Conidial pillar development under these conditions required high RH, temp between 20 and 24 C, and some type of mechanical pressure, such as that provided naturally by the periderm. These laboratory findings agree with the general field observations of Wood and French (13). Conidial pillar development was also achieved in vitro in oatmeal agar cultures when paired ascospore isolates, known to produce conidial streaks, were aseptically macerated, mixed, and introduced into the warm media just prior to gelation.

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