Variation of Hypoxylon pruinatum in Cultural Morphology and Virulence

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Research supported by the College of Agricultural and Life Sciences, University of Wisconsin.
Accepted for publication 26 November 1973.

ABSTRACT

Single ascospore isolates of *Hypoxylon pruinatum* showed extreme variability in cultural morphology and conidial production. Only 5-6% of the isolates were conidial types. The optimum temp for growth of the fungus in culture differed among isolates and also in the same isolate on different culture media. Maximum growth usually occurred at temp from 24 to 28 C. Single ascospore isolates of the fungus showed extreme variability in their virulence to aspen, and virulence was not correlated with rates of growth in culture. Cankers developed readily on plants at temp from 12 to 32 C, although the optimum for canker development varied with the isolate.

Phytopathology 64:663-667.

Hypoxylon canker caused by *Hypoxylon pruinatum* (Klot.) Cke. has been the subject of intensive study by many investigators (3, 4, 6, 8, 10, 12). Many aspects of the disease, however, including the mode of transmission of the causal fungus, the influence of environmental factors on disease, and details of the life cycle of the pathogen, remain to be discovered.

Bier (4) and Oshima (9) reported that single ascospore isolates of *H. pruinatum* differ in morphological characters, growth rates, and the amount of conidial production. Bier (4) derived four distinct cultural types from the eight ascospores in an ascus. Ponomareff (10) found that cultures from conidia grown on malt agar were morphologically similar to those obtained from ascospores and exhibited a wide range of variation. No studies have concerned variation in pathogenicity. Bagga and Smalley (2), in controlled studies, reported that water-stress was necessary for plants to express high susceptibility.

In the present investigations pathogen variability in relation to cultural morphology and virulence was studied.

MATERIALS AND METHODS.—Aspen plants (*Populus tremuloides* Mich.) used in the studies were derived from root cuttings from cankered trees at the University of Wisconsin Arboretum at Madison. Propagation techniques and growing conditions for the production of uniform rooted aspen cuttings have been described (2).

Perithecial stromata as sources for single-ascospore cultures were collected from naturally infected aspens at the University of Wisconsin Arboretum. Techniques for obtaining ascospores, single-ascospore cultures, and cultures from host tissue have been described (2, 5).

To study the variability of the pathogen quantitatively, 200 single-ascospore cultures were prepared and grown for initial comparisons on 2% malt agar. For more detailed studies, the cultural characteristics of five (A, C, F, I, and K) of the 200 single-ascospore isolates, selected to represent the over-all range of variation, were compared under controlled environments on corn meal agar (CMA), potato-dextrose agar (PDA), oatmeal agar (OMA), malt agar (MA), and a synthetic medium containing dextrose, inorganic salts, trace elements, and vitamins (1, 13). Petri plates (25 ml of medium/plate) were seeded with 3-mm diam disks of inoculum cut from the perimeters of 10-day-old cultures grown on 2% malt agar. Eight replications of each isolate on each medium were incubated at 24 C for 14 days in the dark.

The effect of temp on growth and colony morphology of the five selected isolates was compared on the various media inoculated as previously described (five replicates/treatment). The influence of pH on the growth of these same isolates was studied with a liquid basal synthetic medium enriched with vitamins, as previously described (1). Twenty-five ml of each medium were dispensed into 125-ml Erlenmeyer flasks. The pH was adjusted before and after sterilization. Each flask was inoculated with 1 ml of blended mycelium and incubated at the previously determined temp optimum. After 14 days, the cultures were filtered, the mycelium washed, oven-dried, and weighed.

For pathogenicity comparisons, groups of ten, 4-mo-old, greenhouse-grown aspens in 10.2-cm (4-in) diam pots were wound-inoculated as previously described (2) with mycelium from one of five selected single-ascospore isolates (A, C, F, I, or K). Temperature and relative humidity (RH) during this experiment ranged from 22 to 32 C, and 50% and 95%, respectively. Plants inoculated with the various isolates were randomized on the greenhouse bench and watered to field capacity every third day. With this moisture regime under greenhouse conditions, near maximum susceptibility was achieved with minimum plant injury from moisture stress (2). Ten wounded, but noninoculated, plants were used as controls. The lengths of cankers were measured daily for 20 days. The pathogen was reisolated from randomly selected cankered plants.

A more detailed comparison of the effect of temp on canker development was made. Five groups of 48 plants, 64-cm tall, were inoculated with mycelium of one of the selected isolates of the fungus. Groups of 40 plants, eight inoculated with each isolate, were grown at six different temp (12 to 32 C) in growth chambers maintained at 50 to 60% RH. Plants at all temp were watered every 4th day. In the growth chamber a slightly longer watering interval was necessary, as compared to greenhouse conditions, to achieve maximum susceptibility. Five plants with noninoculated stem wounds at each temp were used as controls.

RESULTS AND DISCUSSION.—*Cultural variability*.—None of the authors who studied *H. pruinatum* in culture (4, 9) gave any real indication of the extreme cultural variability shown in single-ascospore isolates of the fungus. In deriving large numbers of cultures from single ascospores, we rarely found two isolates alike. This variability resembled that reported for *Armillaria mellea* (Vahl.) Quel. by Raabe (11), who suggested that the extreme variation in characters of the isolates indicated that the basidiocarp arose from a heterokaryotic mycelium. The majority of our isolates, although variable, were of the mycelial type (conidia never produced or produced sparsely in old cultures). True conidial cultures constituted only 5-6% of all the single-ascospore isolates. Such isolates consisted of slow-growing gray colonies completely covered with conidia. The mycelial isolates varied in rate of growth, color, texture, size, and shape of colony. Selected single-ascospore isolates differing in these various characters are shown in Fig. 1.

The isolates also exhibited a wide range of variation on all media tested (Fig. 2). All the natural media supported good growth of the isolates. Isolates F and I produced the largest colonies on almost all the media tested and they most closely resembled the "wild type" of the fungus isolated from aspen cankers. Isolate C produced conidia on all the media tested.

Influence of temperature and pH.—The optimum temp for growth differed among isolates, and also in the same isolate on different media. Isolate A produced maximum growth at 28 C on the natural media, whereas on the synthetic medium its optimum temp was 24 C. Isolate C produced the largest colonies at 24 C on OMA and synthetic media, although its optimum temp was 28 C on the other media. Isolate F grew best at 28 C on CMA, OMA, and synthetic medium, but on MA its optimum temp was 24 C. Isolate I produced the largest colonies at 28 C on all five media, and isolate K also produced its largest colonies at 28 C on (or) all but the synthetic medium (Table 1).

Different isolates showed different pH optima (Table 2). Growth of all the isolates was negligible below pH 4. In general, good growth occurred over the pH range of 4 to 7. Isolates A, C, and K grew best at pH 7, whereas isolates F and I made the maximum growth at pH 6, but all isolates grew poorly at pH 9.

Pathogenicity.—Isolates of the fungus differed greatly in ability to infect and produce cankers on aspen (Fig.3). No correlation was observed between growth rate in culture and length of cankers produced on aspen. Isolate I, for example, produced the largest colonies on artificial media, but induced only small cankers. Isolate K, which grew sparsely on most media, produced the largest cankers. Isolate C, the slowly developing conidial strain, was nonpathogenic and the inoculated wounds healed with heavy callus production. Isolate F induced cankers

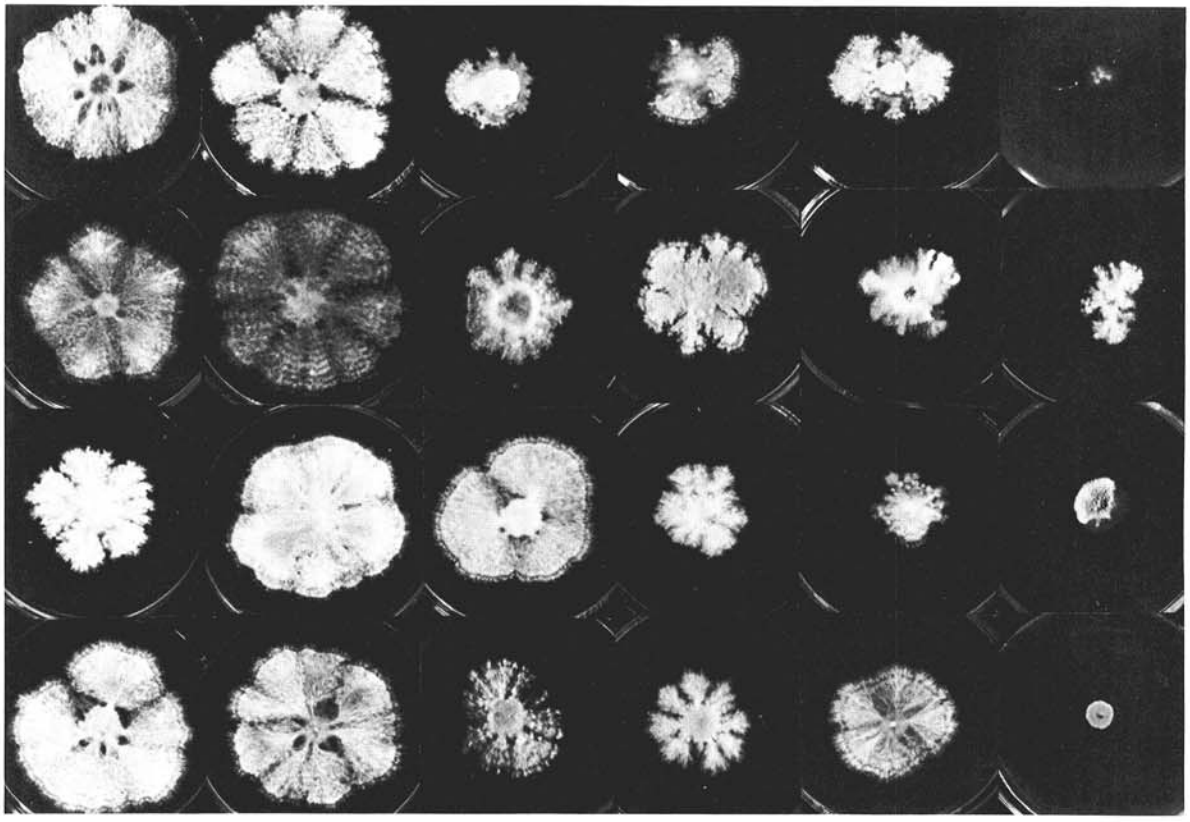

Fig. 1. Typical variability of single-ascospore isolates of *Hypoxylon pruinatum* grown on malt agar for 14 days at 24 C.

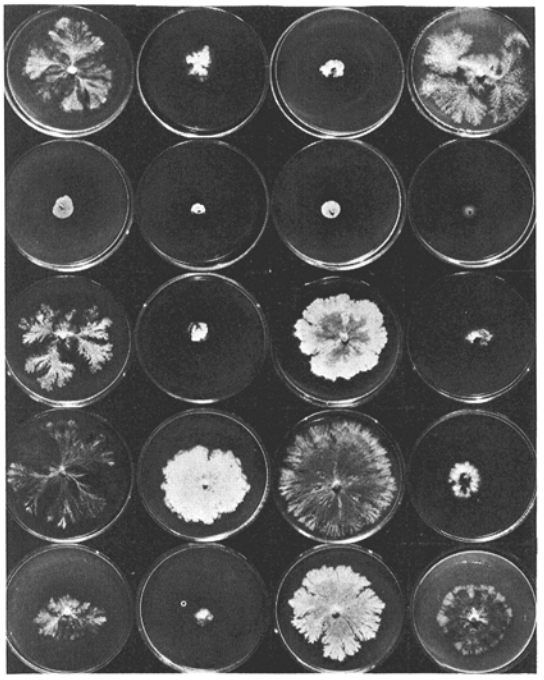

Fig. 2. Cultural characteristics of selected single-ascospore isolates of *Hypoxylon pruinatum* (A, C, F, I, and K) on corn meal agar, potato-dextrose agar, malt agar, and synthetic medium agar after 14 days of incubation at 24 C.

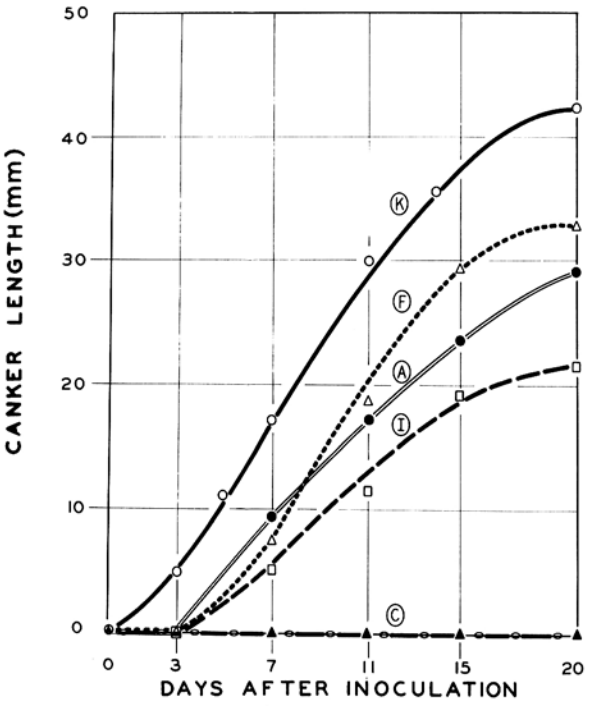

Fig. 3. Rates of canker development of five single-ascospore isolates of *Hypoxylon pruinatum* (A, C, F, I, and K) on inoculated greenhouse-grown *Populus tremuloides* plants. Each treatment was replicated 10 times; plants were watered every third day.

TABLE 1. Linear growth of *Hypoxylon pruinatum* on different media incubated at various temp for 2 wk[a]

Medium	Isolate	Colony diameter (mm) 16 C	20 C	24 C	28 C	32 C
Cornmeal agar	A	trace	41±3	68±6	75±4	29±1
	C	8±0	11±1	14±2	18±3	trace
	F	24±2	24±4	69±5	74±5	40±3
	I	15±1	45±2	75±4	84±3	trace
	K	15±1	21±1	40±4	49±4	47±2
Malt agar	A	10±1	12±1	15±1	20±1	8±0
	C	7±1	8±1	10±1	14±2	0
	F	20±4	32±5	72±5	65±3	54±3
	I	21±3	37±4	78±4	83±2	trace
	K	18±2	45±6	61∓6	70±4	32±1
Oatmeal agar	A	12±1	33±1	69±4	73±2	trace
	C	7±1	8±1	11±2	7±1	0
	F	38±2	45±5	75±7	80±3	40±2
	I	35±3	37±3	80±5	84±3	trace
	K	22±1	23±2	40±4	51±2	27±3
Potato dextrose agar	A	12±1	14±1	19±2	21±1	10±2
	C	5±1	8±2	10±1	13±0	trace
	F	trace	trace	12±1	12±1	9±1
	I	16±2	30±2	61±8	67±2	trace
	K	trace	12±1	14±2	18±3	11±1
Synthetic[b]	A	14±3	20±1	83±5	80±5	12±1
	C	6±1	8±1	12±2	6±1	0
	F	trace	9±1	18±1	27±4	25±3
	I	15±1	18±3	21±1	33±4	11±1
	K	23±2	18±2	47±4	52±5	54±4

[a]Values are means of eight replications.
[b]Synthetic medium contained dextrose, inorganic salts, trace elements, and vitamins.

TABLE 2. Effect of hydrogen-ion concn on the growth of five isolates of *Hypoxylon pruinatum* in a liquid synthetic media 2 wk after inoculation[a]

	Isolate A[b]		Isolate C		Isolate F		Isolate I		Isolate K	
Initial pH	Fungus dry wt. (mg)	Final pH	Fungus dry wt. (mg)	Final pH	Fungus dry wt. (mg)	Final pH	Fungus dry wt. (mg)	Fi pH	Fungus dry wt. (mg)	Final pH
3	trace	3.3	trace	3.3	trace	3.3	trace	3.3	trace	3.3
4	83±5	6.5	67±6	6.2	85±7	6.6	50±5	6.5	50±4	6.3
5	82±9	6.5	65±5	6.2	92±7	6.7	53±3	6.5	59±5	6.8
6	80±4	7.2	67±4	7.3	102±10	7.2	62±4	7.2	68±6	6.9
7	89±4	7.6	68±8	7.5	95±8	8.0	54±3	6.9	74±5	7.5
8	69±5	7.7	50±4	7.6	73±7	7.7	33±3	7.2	72±6	8.3
9	trace	7.7	25±2	7.4	trace	8.7	10±1	8.5	13±2	7.9

[a]Averages of four replications (mg).
[b]Flasks inoculated with Isolates A and C were incubated at 24 C; Isolates F, I, and K were incubated at 28 C.

on all inoculated plants, but the average canker length was less than those induced by Isolates K and A. Isolates A, F, and I did not produce cankers in the first 5 days of inoculation, although four plants inoculated with Isolate K had developed cankers which averaged 11 mm long within 5 days.

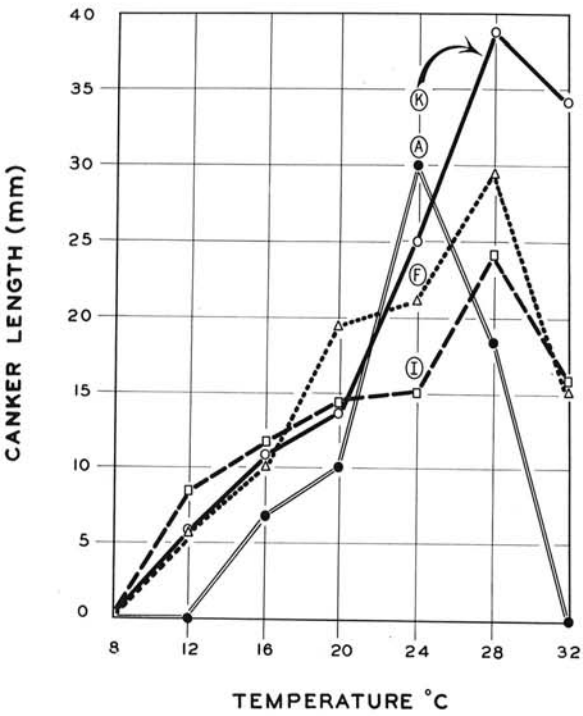

Fig. 4. Effect of temp on canker development on plants inoculated with one of five single-ascospore isolates of *Hypoxylon pruinatum*. Each treatment was replicated eight times; plants were watered every fourth day and harvested after 20 days. No cankers were produced by isolate C at any of the temp.

Cardinal temp for disease development varied with the isolate (Fig. 4). Isolate C was nonpathogenic at all temp. Isolate A produced cankers in a relatively narrow temp range (16-28 C) with an optimum at 24 C. Isolates F, K, and I produced cankers over a broader range of temp (12-32 C) with a maximum number of cankers at 28 C. Noninoculated plants did not become infected.

The great variability of single-ascospore cultures of *H. pruinatum* suggests that no single line really represents the wild type as it can be isolated from aspen cankers in nature. However, several of our isolates, particularly F and I, did resemble the wild type in certain characters. These facts suggest, as Raabe has suggested for *Armillaria* (10), that a heterokaryon wild type must be formed prior to its becoming an active pathogen. This might be accomplished by anastomosing hyphae from masses of ascospores introduced into the infection court through insect transmission. Insect vectors for this disease have been suggested by Bier (4) and Graham and Harrison (7).

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