

STUDIES OF THE BIOLOGY OF HYPOXYLON CANKER OF ASPEN

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## INTRODUCTION

The fungus Hypoxylon pruina<sup>1</sup>tum (Klot.) Cke. causes a canker disease of aspen which can girdle and kill sapling sized trees within 5 years and pole sized trees in 10 to 15 years (3, 7). Populus tremuloides Michx. is highly susceptible to attack, largetooth aspen (P. grandidentata Michx.) moderately susceptible, and balsam poplar (P. balsamifera L.) moderately resistant (7). The disease also has been observed on bolleana poplar (P. alba L. var pyramidalis), on Chinese aspen (P. lasiocarpa Oliver) (3), and on European aspen (P. tremula L.) in Russia (5).

Hypoxylon canker is found on aspen in northeastern United States (36), in the Lake States region (31), in Colorado (19), and in Ontario (7). Anderson (3,4) estimates that the incidence of hypoxylon canker in Minnesota ranges from 1.8 to 6.1 per cent of the standing aspen and that 1 to 2 per cent of the standing aspen volume is killed annually by the pathogen, which exceeds the amount harvested for pulpwood and other uses.

Cankers are found most often in the smooth bark portion of aspen trunks 15 to 40 years old, but cankers also occur on branches (3). Young cankers first appear as yellow-orange, slightly sunken areas of bark, After several months to one year the outer bark layer blisters and sloughs off, exposing the blackened, infected cortex and columnar-shaped hyphal pegs on which conidia are borne. Annual canker enlargement takes place at the average rate of 39 cm. longitudinally and 9 cm. tangentially (44) and callus formation seldom occurs. Approximately

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<sup>1</sup> Tree nomenclature is based on Little, E. L. 1953. Check list of native and naturalized trees of the United States (including Alaska). U.S.D.A. For. Serv. Agr. Handbook No. 41.

3 years after infection the ascigerous stage of the fungus is formed in perithecia embedded in stromata on the older portions of the canker. It is thought that conidia act as spermatizing agents for the formation of the perfect state (37), but positive proof is lacking. The hypoxylon fungus is capable of decaying the wood and predisposing the stems to breakage (33).

The mode of infection in nature is obscure. It is thought that ascospores are the primary inoculum, but so far artificial inoculations with ascospores have failed to produce cankers (7, 20, 38) or have produced them sporadically (25, 44). Graham and Harrison (23) reported a high degree of correlation between wounds made by aspen borers and hypoxylon infection in one area of Michigan. Cankers can be produced artificially when the inoculum consists of mycelium of H. pruinatum (7, 25, 38, 44).

Miller (34), in his recent revision of the genus Hypoxylon, combined H. pruinatum and other species in H. mammatum (Wahl.) Miller ~~comb. nov.~~ This change has not been universally accepted, and further studies are necessary to clarify the relationship between these species.

The objective of this study was to determine the factors involved in spore dispersal and the conditions necessary for the successful establishment of H. pruinatum in the host. Included were studies on the ejection of ascospores from cankers, the wind dissemination of these spores, rate of canker deterioration on dead trees, the possibility of eradication as a control practice, requirements for germination of the ascospores, and the seasonal fluctuation of aspen bark moisture content and its relation to incidence of infection in nature.

## EJECTION OF ASCOSPORES FROM HYPOXYLON CANKERS

The forceable ejection of ascospores is a common occurrence in the Ascomycetes. Bier (7) reported that after perithecia of H. pruinatum were moistened in water, asci passed singly into the ostioles and ascospores were discharged for distances of 2 to 4 cm. Both Bier (7) and Gruenhagen (25) reported that during heavy rains perithecia became soaked with water, and after the rain spores were ejected for more than 25 hours. Wood and French (45) noted that ascospores were ejected in the winter when the water from melting snow was absorbed by perithecia.

In this study several aspects of ascospore discharge were investigated to determine the magnitude of inoculum liberated from aspen infected with H. pruinatum, the duration of ascospore discharge after rains, and the effect that atmospheric conditions had on spore dispersal.

Materials and Methods

These studies were carried out in an aspen stand in Itasca State Park. The aspen were from 20 to 30 feet tall and from 2 to 4 inches diameter breast height (d.b.h.). Underbrush consisted of hazel (Corylus americana Walt. and C. cornuta Marsh.) and viburnum (Viburnum rafinesquianum Schultes) and ground cover of wild sarsaparilla (Aralia nudicaulis L.), large-leaved aster (Aster macrophyllus L.), and poison ivy (Rhus radicans L.). The stand was similar to many aspen stands in the area.

Hypoxylon cankers present in the stand were used and additional cankers were brought into the stand when needed. Only cankers dis-

charging large volumes of spores were used in the study.

Because of the infrequency of rain during the summer of 1963, when this work was done, the ejection of ascospores was initiated by soaking the cankers with water using a portable pump. If the cankers were wetted and spore sampling was done at night, the atmospheric conditions were similar to the conditions existing after a rain. Normally, after a rain the temperature decreased and the relative humidity ranged from 90 to 100 per cent. Without rain, after sundown the relative humidity usually increased to 85 or 90 per cent and the temperature dropped 10 to 40 degrees below the high of the previous afternoon. If a fog was present at night, the relative humidity remained at a high level and conditions were even more similar to postrain weather. It was concluded that by artificially soaking the cankers with water at night, spore ejection from perithecia would be similar to what would occur after a rain, because the temperature and relative humidity in both situations were nearly the same.

A hygrothermograph was placed in a shelter at approximately the same height as the cankers. Temperature and relative humidity were recorded constantly and checked periodically with a thermometer and psychrometer.

Microscope slides were mounted with paper clips over perithecial stromata on cankers to check for ascospore ejection. Black deposits on the slides indicated that spores were being discharged from the perithecia.

A spore sampling machine was located downwind from each canker to obtain a representative sample of the spores liberated from



the entire canker (Fig. 1). The machine used was the rotorod sampler, originally designed by personnel at the Stanford University Aerosol Laboratory (1) (Fig. 2).

The rotorod sampler was used because it was better suited for field use than were other types of samplers and because it was designed to collect air-borne particles as small as 20 microns with high efficiency. Asai (6) used this sampler to collect urediospores of Puccinia graminis tritici. H. pruinatum ascospores average 26 by 11 microns in size. The rotorod sampler combined the accuracy of a motor-driven sampler which pumped a precise amount of air through a collector and the simplicity of design of glass slide exposures. Instead of relying on wind movement or pumped air to bring air-borne particles to the sampler, energy was applied to the collector which moved through the air. Particle collection was achieved by impacting the air-borne particles on the leading surfaces of a pair of upright brass collectors, 0.16 cm. wide and 6 cm. long, rotating at a distance of 4 cm. from the axis of rotation.

The spore collectors were rotated by a DC-powered motor. The drive motor contained a speed-control mechanism which maintained maximum rotation rate at 2500 r.p.m. with linear velocity at 10.6 m/sec. (23.5 m.p.h.) with a battery voltage of 10 volts or higher (1). The flow rate of the sampler, defined as the volume of air swept out by the leading surface area of the collectors per unit time (or the volume of air sampled), was 120 liters per minute. This was reduced by only 5 per cent at wind speeds of 11 m.p.h. Compared with other samplers, the rotorod sampler sampled up to 20 times as much air volume per



Figure 1. On the left, a rotorod sampler located downwind from a hypoxylon infected aspen. The portable pump in the center was used to moisten the canker to initiate the ejection of ascospores.



unit time, and this feature was important in the present study for determining the magnitude of ascospores liberated from cankers during specific time intervals.

Rotorod samplers also were used in later studies dealing with the movement of ascospores in a deciduous stand and the entrance of spores into an isolated aspen stand.

#### Rotorod Sampler Methodology

The techniques employed in using the rotorod samplers were fundamentally the same as those described in the report by the Aerosal Laboratory at Stanford University. Modifications were taken from Harrington, et al. (26). The procedures were as follows.

A sticky substance was applied to the collector bars where the spores were to be impacted (Fig. 3A). The adhesive used exclusively in these studies was a mixture of one part rubber cement and two parts xylol. This mixture was toxic to the spores and prohibited subsequent culturing of the fungus. A non-toxic gelatin-glycerin mixture could have been used as an alternative but its adhesive properties were adversely affected by moisture and storage. The rubber cement-xylol adhesive was allowed to dry while the bars were in an inverted position. This enabled excess adhesive to drip from the bars and resulted in a more even coverage of the bars. The collector bars were stored on a rack and several racks of bars were carried to the field in a specially designed case (Fig. 4).

The sampler could be operated in either a clockwise or counterclockwise direction, thereby collecting two samples per rod. Depending on the amount of dirt particles, spores, pollen grains, and insects

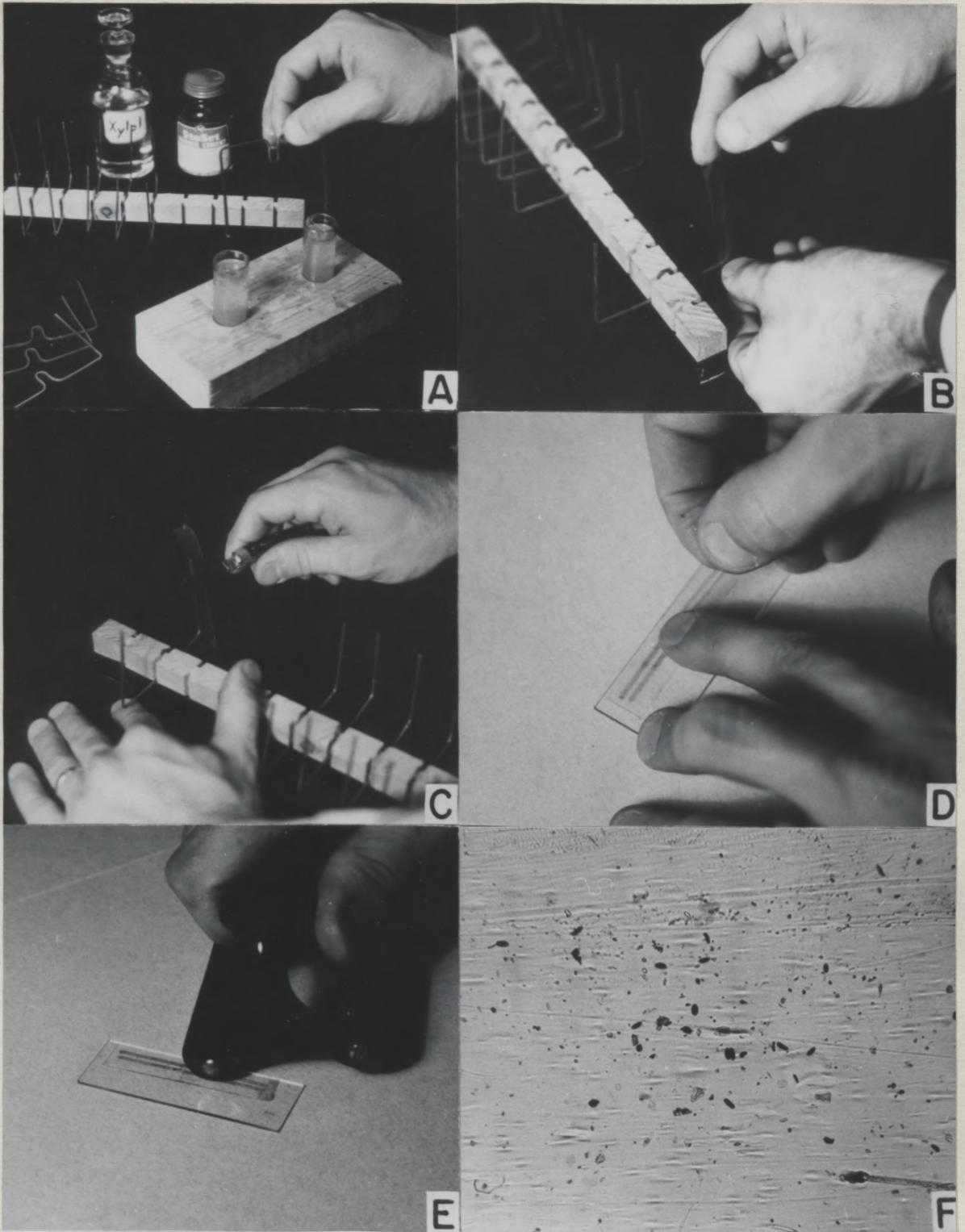


Figure 3. Procedures for preparing the rotorod collector bars and for processing the sampled material. A. The bars were coated with a 1:2 mixture of rubber cement and xylol and dried. B, C. Cellulose (Scotch) tape was applied to the two leading surfaces of a bar which had been run in a clockwise direction. D. The tape was removed and placed on a microscope slide. E. A roller smoothed out the tape and eliminated most of the air bubbles which would interfere with counting the spores. F. The finished slide showing ascospores magnified 90X.

in the air, exposure time per rod varied from one-half to several hours. The greater the number of particles per rod the more difficult it was to analyze the sample. After a rain, samplers were run for 3 hours with little particle collection because the air was reasonably clear.

After collecting the sample the adhesive substance on the rods containing the air-borne spores was removed and transferred



Figure 4. Equipment used in the field for spore sampling. The specially designed carrying case contains 6 racks of collector bars. Microscope slides were mounted on cankers to check for ascospore ejection from individual stromata.

the microscope was not difficult due to the characteristic shape and dark color of the spores. Species of the genus *Hypoclytus* with ascospores of similar appearance as those of *H. prasinatum* may have been present on the slides, but they could not be distinguished, and all spores which looked like *H. prasinatum* ascospores were counted as such. When using artificially inoculated cankers it was not likely that

in the air, exposure time per rod varied from one-half to several hours. The greater the number of particles per rod the more difficult it was to analyze the sample. After a rain, samplers were run for 8 hours with little particle collection because the air was reasonably clean.

After collecting the sample the adhesive substance on the rods containing the air-borne particles was removed and transferred to a microscope slide. This was done by applying cellulose (Scotch) tape to each of the two leading surfaces of the collector rod which removed the adhesive substance (Fig. 3B, C, ), and the tape was smoothed out on a microscope slide (Fig. 3D). Air bubbles under the tape interfered with spore counting but a roller was used to eliminate most of these bubbles (Fig. 3E).

The air-borne particles that adhered to the collector bars could be observed on the slide directly through the cellulose tape with a compound microscope (Fig. 3F). The width of the collector bar and the subsequent width of the sample material on the slide were the same as the diameter of the field of vision when viewed at a magnification of 100X.

Distinguishing hypoxylon spores from the other material under the microscope was not difficult due to the characteristic shape and dark color of the spores. Species of the genus Hypoxylon with ascospores of similar appearance as those of H. pruinatum may have been present on the slides, but they could not be distinguished, and all spores which looked like H. pruinatum ascospores were counted as such. When using artificially moistened cankers it was not likely that

other species would be air-borne.

Storage of air-borne particles on microscope slides has several advantages. Samples do not have to be analyzed immediately after collection, few pieces of equipment need be taken to the field to collect and preserve the samples, and samples can be reviewed years later. Slides have been kept in this laboratory for 2 years and the spores still counted with ease.

Although the air volume swept past the collector bars of the rotorod sampler is calculated to be 120 liters per minute with a battery voltage of more than 10 volts (1), the exact air volume was not determined in these studies. Equipment for measuring the rotation rate of the collector bars was not always available so that changes in motor speed could not be detected.

### Results

One part of the study involved soaking the cankers once and checking for ascospores until spore ejection stopped. On four occasions sampling was started at 8 P.M., 10 P.M., midnight, and 2 A.M. Slides and spore samplers were changed every hour. The results are shown in Figures 5-8.

No matter when during the night cankers were soaked with water spore ejection always terminated or was approaching termination by noon of the following day. When sampling was continued into the afternoon no spores were collected by the rotorod samplers nor were spores deposited on the slides mounted on cankers. The constant decrease of numbers of spores collected by the samplers from about 7 A.M. until noon closely followed the morning change in atmospheric



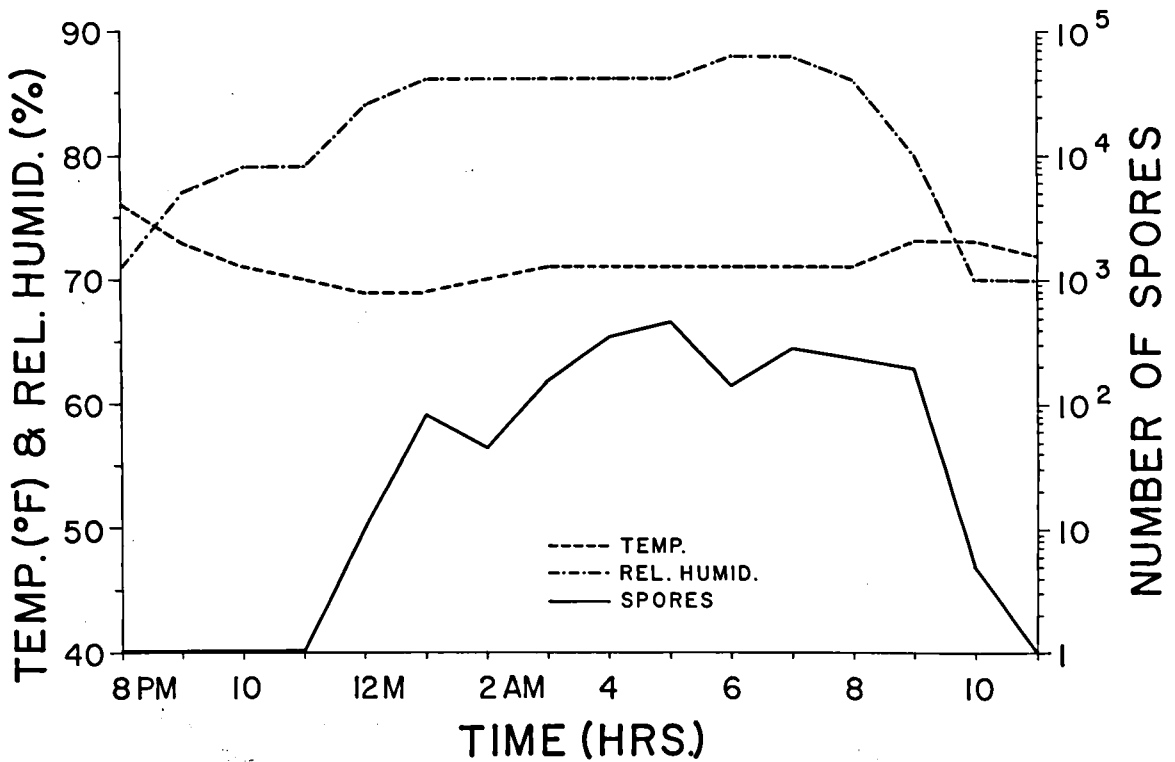


Figure 5. Numbers of ascospores collected by a rotorod sampler from one canker moistened at 8 P.M. on 8/22/63. The canker had not been wet for 12 days prior to the study.

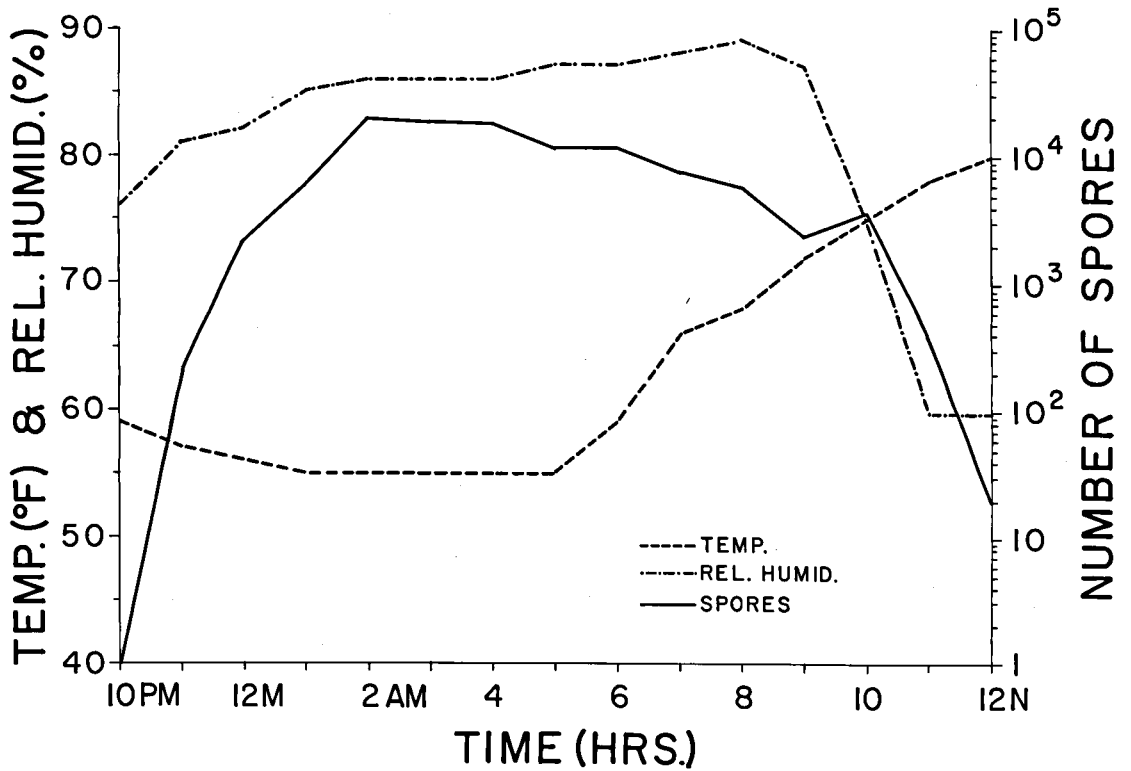


Figure 6. Numbers of ascospores collected by rotorod samplers from cankers moistened at 10 P.M. on 7/19/63. The cankers had not been wet for 6 days prior to the study. The graph represents the average data from 4 cankers.

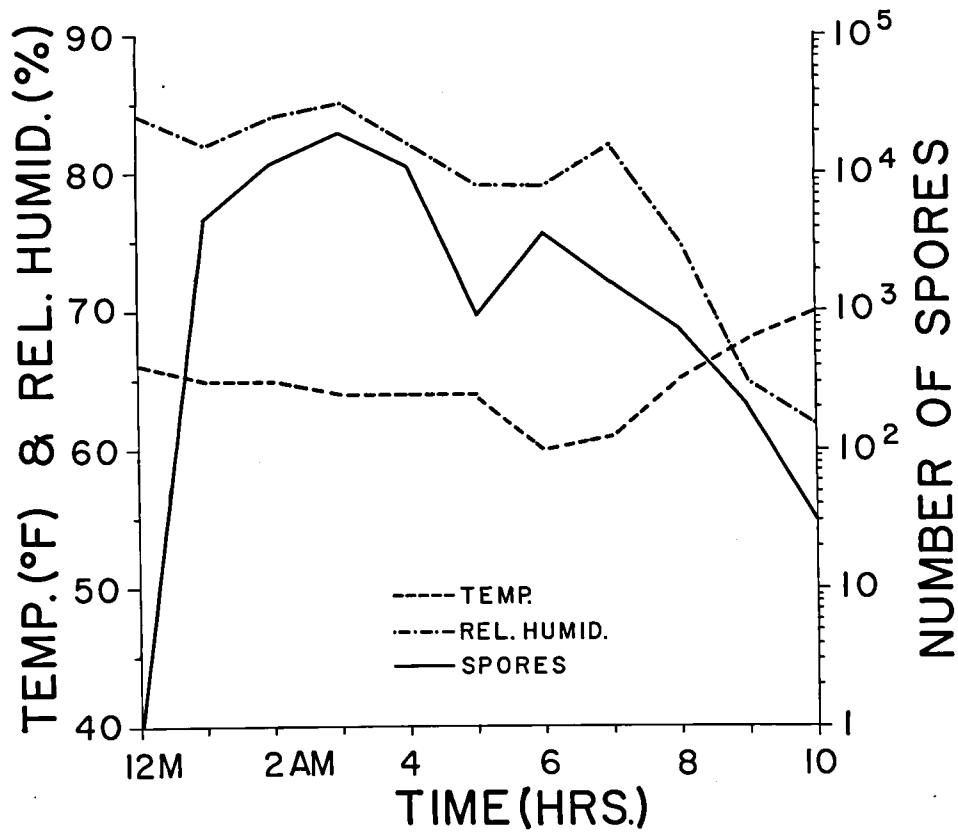


Figure 7. Numbers of ascospores collected by rotorod samplers from cankers moistened at midnight on 7/13/63. The cankers had not been wet for 2 1/2 days prior to the study. The graph represents the average data from 4 cankers.

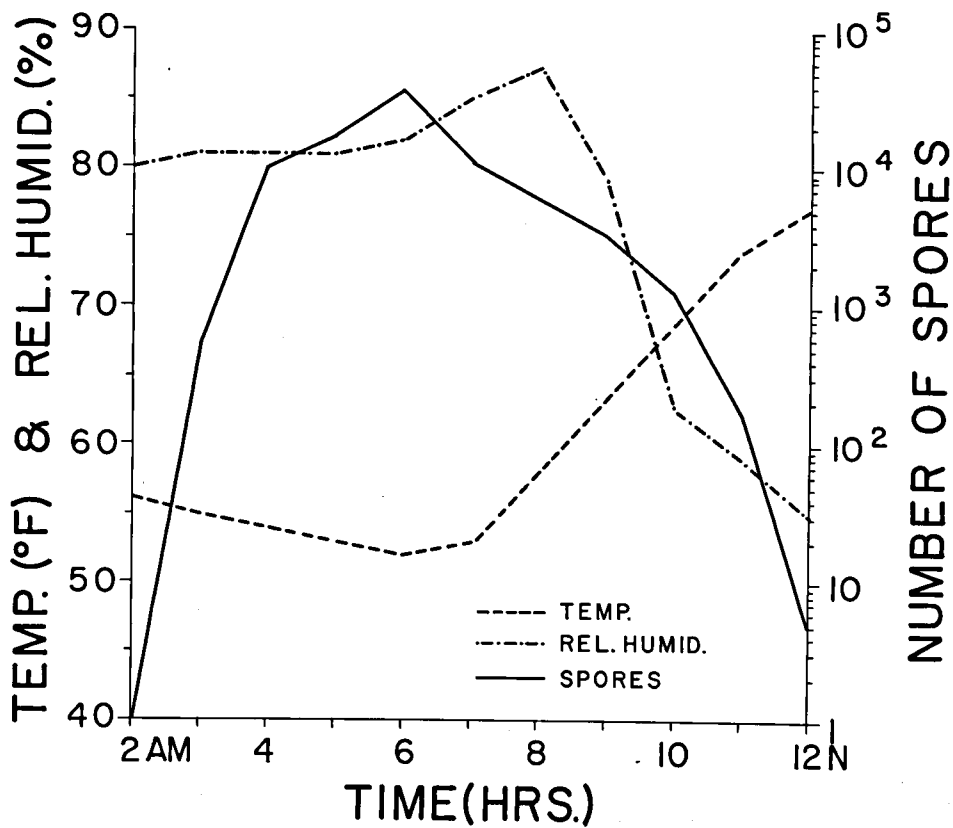


Figure 8. Numbers of ascospores collected by rotorod samplers from cankers moistened at 2 A.M. on 7/10/63. The cankers had not been wet for 5 days prior to the study. The graph represents the average data from 4 cankers.

conditions, especially the decreasing relative humidity. Maximum relative humidity and minimum temperature for the day normally occurred between 6 and 8 A.M. After this time, temperature rose rapidly with a corresponding drop in relative humidity. Occasionally the largest <sup>nt</sup> quantity of spores was collected by the samplers between 6 and 8 A.M. The cankers appeared to be dry by noon.

The largest number of spores for any 1-hour period was liberated about 4 hours after the cankers were initially wetted (Fig. 6 and 8). However, this time period varied. The period of maximum spore discharge occurred from 1 to 9 hours after soaking the cankers, depending on the previous moisture condition of the cankers. If the cankers had been recently wetted (within the previous 1-3 days), ascospore ejection reached a high level within the second or third hour of sampling (Fig. 7). If the cankers had been dry for at least 10 days prior to sampling, maximum spore dispersal occurred after 8 or 9 hours (Fig. 6). Coupled with delayed spore liberation the total volume of spores collected from these cankers was small.

The longest period during which spores were collected in this type of study was 18 hours. This record occurred with one canker moistened at 7 P.M. Whereas other cankers in the same trial discontinued ejecting spores at 10 A.M. the following morning, this one canker continued to liberate spores until approximately 2 P.M.

Spore deposits on microscope slides placed over a canker indicated the pattern of spore ejection from all the stromata on that canker. There was usually a close correlation between the mass of spores on the slides and the number of spores collected by the rotorod sampler for a given 1-hour period. Table 1 shows that spore deposits from these

three locations on trees 1 and 2 are uniform in magnitude for given 1-hour exposures, becoming larger in size then smaller with the increase and decrease of spores liberated from the cankers. The correlation was not as close for trees 3 and 4, but the same general pattern prevailed.

By comparing patterns of spore deposits on slides for consecutive 1-hour periods it was possible to follow spore ejection from a single perithecium. Figure 9 shows the mass of spores from one location on a canker for a period of 15 hours. Spores were ejected from one perithecium in large enough quantity to develop a visible spore mass on each slide, exposed for 1 hour, for a total of 9 consecutive hours.

In another part of the study, the same cankers were moistened at 3-hour intervals for periods up to 96 hours to test for spore ejection over extended periods of time. The results of one trial are presented in Figure 10.

Spore ejection was directly proportional to changes in relative humidity and inversely proportional to changes in temperature for the first 24 hours of the study. After 24 hours spore counts did not follow the changes in atmospheric conditions as closely, although spore counts from individual trees were similar to one another for 57 hours.

Whereas changes in relative humidity and temperature followed a 24-hour cycle, spore liberation followed a 12-hour cycle. Maximum spore liberation during each 24-hour period of the 96-hour study occurred at 10 P.M. By soaking the cankers with water every 3 hours

Table 1. A comparison of the number of spores collected by rotorod samplers located downwind from 4 cankers and the spore masses deposited on glass slides mounted over perithecial stromata on the same cankers. The cankers were soaked with water at 2 A.M. on 7/10/63. The cankers had not been wet for 5 days prior to the study.

Hour	Tree No. 1			Tree No. 2			Tree No. 3			Tree No. 4		
	Spore Count	Slide A	Location B C	Spore Count	Slide A	Location B C	Spore Count	Slide A	Location B C	Spore Count	Slide A	Location B C
3 A.M.	106	**	* *	780	*	- *	420	-	- -	840	-	- -
4	18,120	***	*** **	10,800	**	** **	7,800	*	* -	3,420	-	- -
5	16,140	***	*** **	17,400	**	** **	12,720	**	*** *	14,820	-	** -
6	37,560	**	*** **	28,500	**	*** **	30,000	**	*** *	44,640	*	** *
7	10,260	**	** **	10,800	**	** **	5,100	*	* *	14,760	-	* *
8	9,060	**	*** **	4,980	**	** **	1,440	**	** *	8,460	-	* *
9	3,480	*	* **	1,680	**	** **	5,520	**	** *	2,400	-	* *
10	930	*	- *	780	*	** *	3,240	*	* *	164	-	* -
11	72	-	- -	106	-	* -	420	-	- -	20	-	- -
12 noon	3	-	- -	10	-	- -	6	-	- -	0	-	- -

- = no spores macroscopically visible; \* = spore masses barely visible macroscopically; \*\* = spore masses easily visible macroscopically; \*\*\* = spore masses prominent macroscopically.

## TREE NO. 8 LOCATION A 8/22-23/63

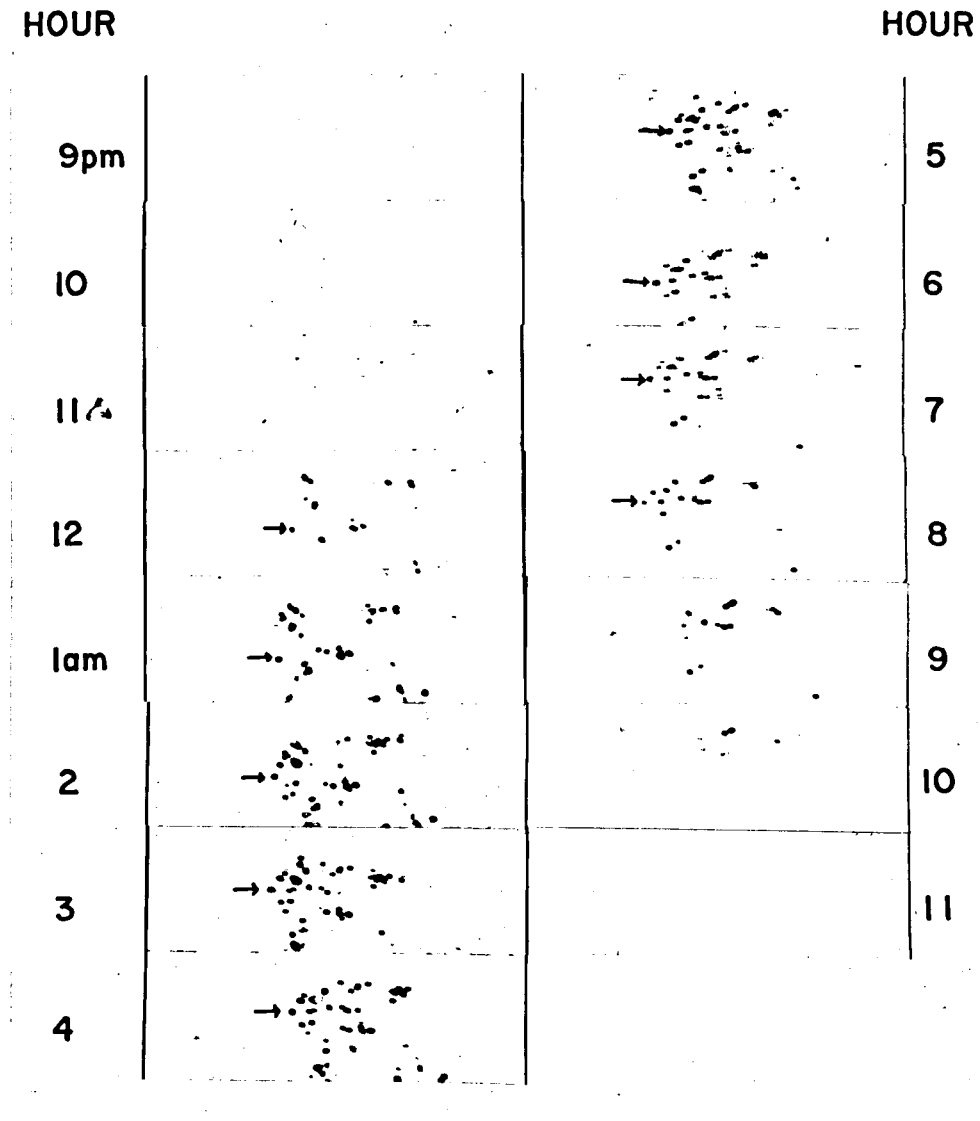


Figure 9. Ascospore deposits on glass slides mounted for consecutive 1-hour periods at one location on a canker. The canker was soaked with water at 8 P.M. and had not been wet for 12 days prior to the start of the study. The arrows point to spore deposits from the same perithecium.



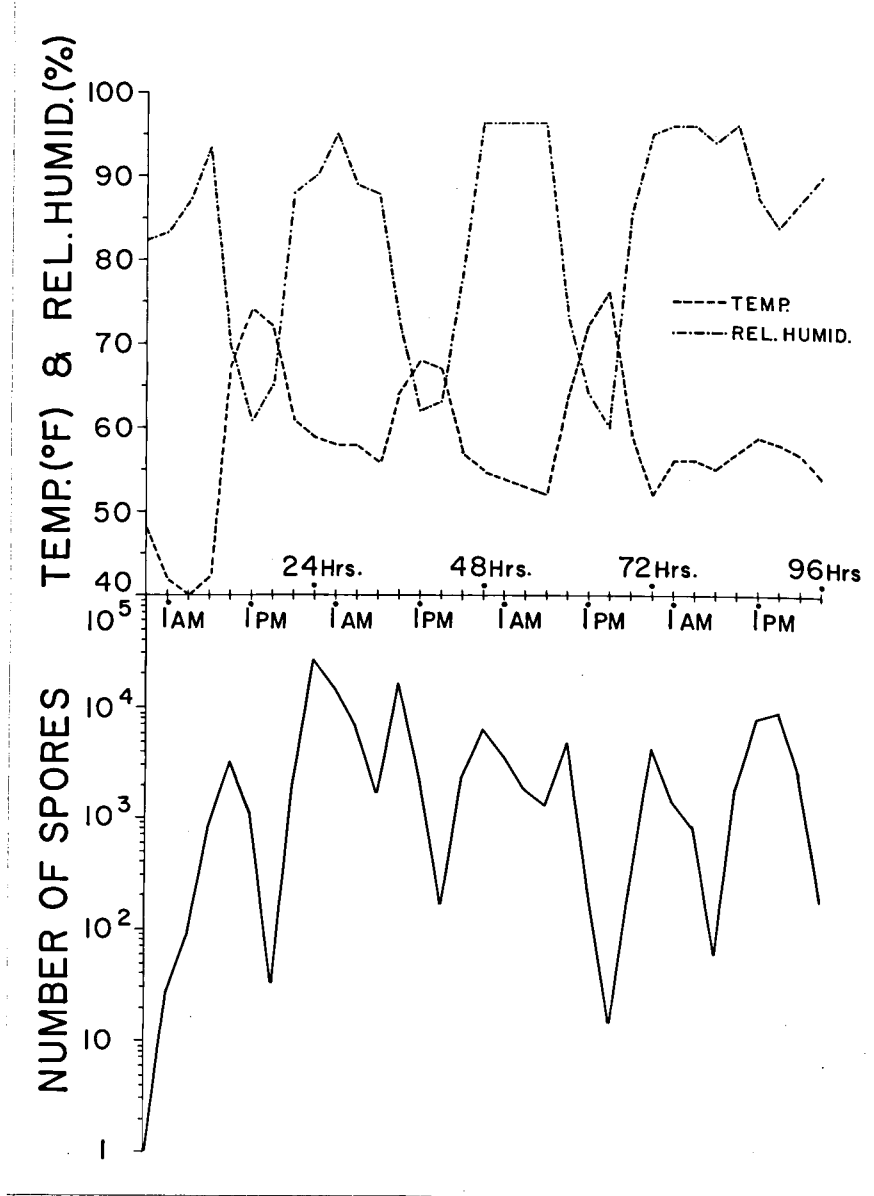


Figure 10. Numbers of ascospores collected by rotorod samplers from cankers moistened at 3-hour intervals for 4 days starting at 10 P.M. on 10/16/63. The graph represents average data from 4 cankers until hour 57 and from 2 cankers from hour 57 through hour 96. A fog developed in the area after 72 hours and remained for the following 12 hours.

secondary peaks of spore liberation were produced at approximately 10 A.M. each day. Minimum spore dispersal occurred at 4 P.M. each day, but decreased spore dispersal also was recorded at 7 A.M. The times of maximum and minimum spore dispersal in a 24-hour period corresponded to the times of maximum and minimum relative humidity during that same period. However, the secondary increases and decreases of spore collections did not correspond to similar fluctuations of relative humidity for the same times. The secondary peaks of spore dispersal were produced when the relative humidity was decreasing and the temperature was increasing. The slight decreases in spore liberation resulted when the relative humidity was still quite high and the temperature relatively low.

The third night of the trial a dense fog developed and remained in the area until the fourth day. It's effects on spore liberation were indicated by an increase in spore counts the morning of the fourth day. Previous to this, spore counts had been steadily dropping since the first day. The fog apparently kept the cankers wet during the daylight hours when normally they would have dried out, and the rhythmic cycle of spore liberation, established during the first three days, was altered.

Figure 11 shows the pattern of spore deposits for 96 hours from one stroma containing about 30 perithecia. Ejection from individual perithecia can be followed for the duration of the study, indicating that perithecia continue to produce spores during conditions which favor spore ejection.

TREE NO.1 LOCATION C  
10/17/63

	7	4
HOUR		
1am	10	7
4	1pm	10
7		
10	7	4am
1pm	10	7
4	1am	10
7	4	1pm
10	7	4
1am	10	7
	1pm	10

Figure 11. Spore deposits on microscope slides mounted at one location on a canker. The canker was wetted at 3-hour intervals starting at 10 P.M. on 10/16/63. The large spore mass in the center of the slides represents deposits from one stroma. The arrows point to deposits from one perithecium.

## Discussion

Hypoxylon cankers, soaked with water when relative humidity and temperature were similar to conditions after a rain, ejected ascospores for periods up to 18 hours. These favorable atmospheric conditions occurred most often at night. Although ascospore ejection was not initiated by 100 per cent relative humidity (25), relative humidities in the high 80's and 90's did influence the length of time that ascospores were ejected after the cankers had been initially moistened. Cankers moistened at 8 or 10 P.M. ejected spores for longer periods of time than did cankers which were moistened at midnight or 2 A.M. This was because the cankers moistened earlier were exposed to the higher relative humidities and stayed wet for a longer time. No matter what time at night the cankers were soaked with water, ascospore liberation decreased when the relative humidity decreased after 8 A.M. the following morning.

On the other hand, when free moisture was in the air, as with a fog, moisture could be absorbed by perithecial stromata and spores ejected. During the fourth day of the study shown in Figure 10, microscope slides mounted on cankers lying on the ground contained spore masses. The only source of moisture for these cankers came from the fog present in the area the night of the third day and the morning of the fourth day. Free water in the air also influenced spore liberation from cankers on standing trees. In the same study (Fig. 10), the fog apparently was responsible for altering the cycle of spore liberation established by the cankers during the previous three days.

The moisture condition of a canker before a simulated rain determined the time lapse between the end of the "rain" and the beginning

of ascospore liberation as well as the volume of spores liberated. Cankers recently moistened ejected large volumes of spores within 1 or 2 hours after being remoistened (Fig. 7). Cankers dry for 10 or more days prior to wetting did not liberate large volumes of spores for 8 or 9 hours. Either the perithecia on these cankers did not contain many spores to start with or, more probably, the cankers did not absorb the optimum amount of water necessary for large volume spore ejection before the warm dry morning atmosphere ended all chances of continued spore liberation.

Individual perithecia could eject spores for at least 9 consecutive hours after a simulated rain once spore liberation was initiated. In Figure 9 the same perithecia contributed spores to the pattern for the majority of the 12 hours that spores were deposited on the slides. However, some perithecia discontinued ejecting spores during the early hours of the study whereas other perithecia began ejecting spores later. This seemed to indicate that perithecia in neighboring stromata were in different stages of maturity. Mature perithecia probably contributed spores for the longest periods of time and newly mature and older perithecia only for short periods.

Spore ejection from cankers moistened at 3-hour intervals for 4 consecutive days reached a peak after 24 hours with nightly peaks decreasing in magnitude thereafter (Fig. 10). Under these constant wet conditions maximum spore ejection occurred at 10 A.M. and 10 P.M. Minimum spore ejection occurred at 7 A.M. and 4 P.M. Within one 24-hour period the largest number of spores were collected in the samplers at 10 P.M. , which was approximately the time of

highest relative humidity for that period. The least number of spores was collected at 4 P.M., which approximated the time of lowest relative humidity for the 24-hour period. Secondary minimum and maximum levels of spore liberation at 7 A.M. and 10 A.M., respectively, both occurred when the relative humidity was decreasing and the ambient temperature was increasing. The ejection of ascospores appeared to be influenced by relative humidity and temperature at certain times and by some unknown factor at other times.

One possible explanation for the above findings is based on the constant buildup of spores in the perithecia. From the present study, and other studies, it appears that ascospores are formed and mature in perithecia only when the canker is moist. If this were not true, then cankers ejecting spores after long dry periods would liberate more spores than cankers which had been dry for only a few days. (Compare Figures 5 and 7). In order for the 12-hour cycle of spore ejection to be produced in Figure 10 there would have to be a constant production of spores in the perithecia. Because of the position of the peaks in the graph the rate of spore formation seems to be constantly decreasing with time. A 24-hour segment of the graph includes a large decrease in spore liberation (4 P.M.) followed by a large increase in liberation (10 P.M.); and this is followed by a smaller decrease in spore liberation (7 A.M.), with a small increase (10 A.M.). The cycle is then repeated, If spores are formed in the perithecia at a relative constant rate, a cyclic graph of spore liberation would result if spore ejection were influenced both by the relative humidity of the surrounding air (influencing rate of canker drying) and by the number of spores in the perithecia

(creating pressure due to crowding). This being the case, large volumes of spores would be liberated both when the atmospheric conditions were optimum for ejection and when the pressure within the perithecia became great enough, due to crowding of asci, so that spores were ejected even when atmospheric conditions were not optimum.

## MOVEMENT OF ASCOSPORES IN A DECIDUOUS FOREST

The ascospores of H. pruinatum are wind disseminated. The forceful ejection of these spores from fruiting bodies exposes them to air currents within the stand and to mass air movements between stands. The presence of hypoxylon cankers in isolated aspen stands indicates that the pathogen can be carried considerable distances by the wind and is not limited to local dispersal.

There are several references on air movement in and around deciduous stands (21, 29, 43, 46), and in aspen forests in particular (32), but no reports were found regarding the movement of fungus spores through forest stands from a known source. The purpose of this study was to follow the movement of ascospores of H. pruinatum by using rotorod samplers. Van Arsdel (41) determined the pattern of sporidial movement of Cronartium ribicola from Ribes spp. to white pine by observing the dispersal of smoke by air currents, but he did no quantitative studies of the spores in the air.

Materials and Methods

Since hypoxylon canker is found to some extent in all the aspen stands in the Itasca Park area, these stands were not used in the study because of the possibility of the presence of H. pruinatum ascospores in the air from sources other than the known source. The stand selected consisted of paper birch (Betula papyrifera Marsh.), ash (Fraxinus nigra Marsh. and F. pennsylvanica Marsh.), and elm (Ulmus americana Michx.), with balsam fir (Abies balsamea (L.) Mill.) scattered throughout the stand. The nearest hypoxylon infection was at least 2000 yards



distant and was not upwind during the trials.

The study area was located on a peninsula about 1000 yards wide extending into a small lake. Optimum wind direction for the study was from the WNW, which came directly off the lake, across the peninsula, and onto a boggy area to the leeward side. The study area was situated near the leeward side of the peninsula when these wind conditions prevailed.

Hypoxylon cankers, capable of ejecting ascospores when the cankers were moistened, were removed from standing trees and brought to the study area. The cankers, which served as a source of spores, were leaned against a fallen log. Twenty rotorod samplers were located downwind from the spore source, beginning directly in front of the cankers and extending out to a distance of 180 ft. (Fig. 12). Ten samplers were 3 ft. above the ground and 10 were fastened in trees 20 to 23 ft. above the ground.

Sampling was conducted on non-rainy days when H. pruinatum spores from other sources would not be present in the air. Cankers were moistened and samplers were operated after 9 A.M. and before 4 P.M., with individual sampling periods not exceeding 4 hours during any one trial.

### Results

The results from one trial, presented in Table 2, were obtained when the wind exceeded 10 m.p.h. outside the stand with gusts to 25 m.p.h. Inside the stand air movement was considerably less. No hypoxylon-like spores were collected prior to when the cankers were moistened.

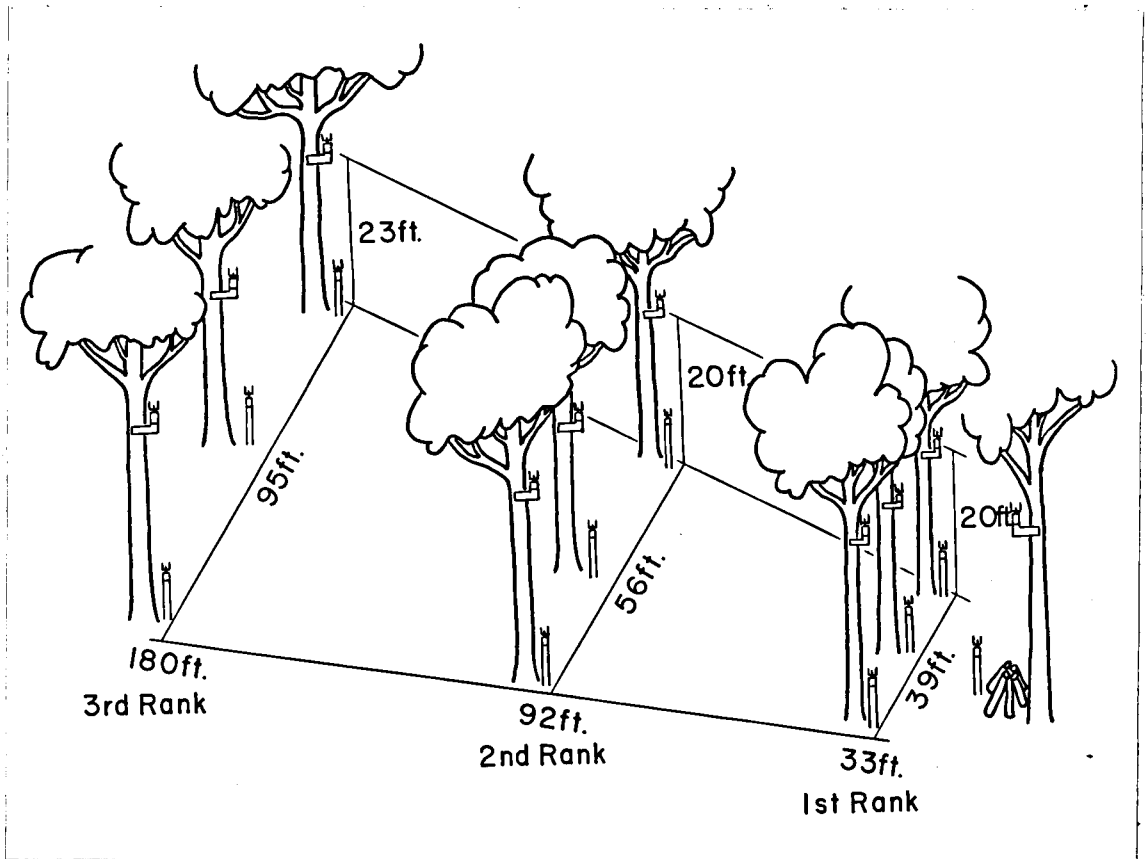


Figure 12. Diagram of the site used in studying the movement of ascospores, in a deciduous stand, from a known source. The spore source is located on the right. Optimum wind for sampling came from the WNW which moved from right to left across the diagram. The base of the canopy was about 40 ft. from the ground and the space between the ground and the canopy was relatively free of vegetation.

Table 2. Numbers of ascospores collected by rotorod samplers in a deciduous stand as the spores moved downwind from a known source. Sampling was continuous from hour 1 through 4 except for an interval between hours 2 and 3 when the sampling bars were changed. Sampling locations correspond to those in Fig. 12.

Distance from spore source (ft.)	Sampler height (ft.)	Number of Spores				Total spores #
		Sampling Hour				
		1	2	3	4	
3	20	0	0	0	0	0
3	3	863	1,279	1,721	1,619	5,482
33	20	1	1	0	1	3
33	3	164	295	214	97	370
33	20	1	0	1	0	2
33	3	38	139	97	78	352
33	20	1	1	0	0	2
33	3	0	0	2	9	11
92	20	0	0	1	0	1
92	3	9	33	26	10	78
92	20	0	1	0	0	1
92	3	7	4	19	9	39
92	20	0	0	0	0	0
92	3	1	0	0	4	5
180	23	1	0	2	0	3
180	3	0	0	0	0	0
180	23	0	0	1	3	4
180	3	0	1	7	0	8
180	23	0	0	3	1	4
180	3	0	1	3	3	7
		1,086	1,755	2,097	1,834	6,772

In this study the number of spores collected by the samplers decreased rapidly with increased distance from the spore source. Compared with collections at 3 ft. in front of the spore source, average spore dilutions at 33 ft., 92 ft., and 180 ft. were 1:5, 1:44, and 1:210 respectively. In other studies, spore dilution at 180 ft. ranged from 1:100 to 1:1000.

Results from 4 separate studies are in Table 3, the data given in spores per hour and per cent of spores in upper and lower samplers at each distance from the source. The principle variable between the studies was the wind velocity outside the stand. Wind speeds greater than 10 m.p.h. resulted in spore collections similar to those in Table 2. With wind speeds of less than 5 m.p.h. more spores were collected from the upper samplers at the closest collection point, 33 ft. from the source. Per cent collections in the other samplers were approximately the same for all 4 studies.

The higher percentage of spores collected in the upper samplers at 33 ft. from the spore source indicated that air currents rapidly rose a short distance from the source when wind speeds were less than 5 m.p.h. Orange smoke was released from near the cankers during various wind speeds to observe air currents and possible patterns of spore dispersion. Winds greater than 10 m.p.h. outside the stand caused the smoke within the stand to remain close to the ground until past the first set of samplers at 33 ft. It then rose extending from the ground to about a height of 20 ft. at 92 ft. from the source. Thereafter, vertical and horizontal dispersion increased rapidly. The smoke was barely visible at the third rank of samplers 180 ft. from the source.

Table 3. Ascospore collections from four separate studies during high and low wind velocities. Data are presented in average number of spores per hour and per cent of spores in upper and lower samplers at each distance from the source of spores.

Distance from spore source (Ft.)	Sampler height (Ft.)	Wind greater than 10 m. p. h.				Wind less than 5 m. p. h.			
		Test I		Test II		Test III		Test IV	
		Spores	%	Spores	%	Spores	%	Spores	%
3	20	0	0	0	0	0	0	0	0
3	3	1370	100	1219	100	2100	100	8400	100
33	20	2	1	6	1	74	29	77	8
33	3	283	99	886	99	181	71	855	92
92	20	1	3	12	8	1	7	15	8
92	3	30	97	135	92	13	93	182	92
180	23	3	43	14	42	2	50	29	41
180	3	4	57	19	58	2	50	42	59

Winds less than 5 m.p.h. allowed updrafts within the stand to carry smoke from the source to above a height of 20 ft. before the smoke had moved 33 ft. laterally downwind. Some updrafts carried smoke up to the canopy. Beyond the samplers located 33 ft. from the source, vertical and horizontal dispersion of the smoke was approximately the same regardless of wind velocity.

### Discussion

In this study, rotorod samplers were capable of trapping H. pruina-  
tum ascospores moving from a known source up to a distance of at least 180 ft. The dilution factor at this distance was great enough to discourage sampling at greater distances. If a larger concentration of spores had been released at the source, it is possible that samplers located further away than 180 ft. would have collected spores. The concentration of spores in the air could be calculated if the exact speed of rotation of the sampling bars was known; however, this was not known for this study. The bars are capable of sampling 120 liters of air per minute at maximum r.p.m. with wind velocity less than 11 mph (1).

Different patterns of spore dispersal were observed depending on wind velocity. By comparing spore collections with the movement of colored smoke, it was concluded that the movement of ascospores was subject to minor air currents in the stand in addition to mean wind direction. Wind speeds in excess of 10 m.p.h. outside the stand caused both spores and smoke to travel without much vertical and horizontal dispersion until past the samplers located 33 ft. from the spore source, and finally leave the stand below the canopy. Slower winds allowed spores and smoke to gain altitude rapidly due to local air currents in the

stand. Sometimes the smoke traveled at a steep incline to the canopy and moved above the trees at a horizontal distance of 30 to 60 ft. from the source. On one occasion the smoke just below the canopy changed altitude several times in serpentine fashion while moving away from the source. Hypoxylon spores riding the same air currents as the smoke in the above two situations could leave the stand through the canopy shortly after ejection from a canker and could possibly be carried long distances by air movement above the stand.

Exiting the stand through the top of the canopy could be of great significance to the dissemination of inoculum. The higher the spores can get before encountering prevailing winds the greater the probability of long ranged dissemination. Spores leaving the stand below the canopy at the leeward margin of the stand probably would not be disseminated as far. Gregory (24) stated that 99.9 per cent of the spores should fall to the ground within the first 100 meters from a source lying on the ground. His conclusions, however, were based on dissemination in an open field and did not account for updrafts. Zentgraf and Eisenkolb (46) reported that wind speed acceleration was noted toward the leeward margin of a deciduous stand. This was ascribed to a suction effect exerted by the air flowing over the canopy and sinking as it left the leeward margin. Such an effect would no doubt discourage the gain in altitude of spores which had not already left the stand through the canopy. On windy days it is doubtful that spores can leave the stand other than below the canopy at the leeward margin due to the lack of updrafts in the stand. On windy days, Gregory's conclusion, stated above, might apply. Beside the lack of local updrafts in the stand to carry spores through the

canopy, the suction effect would keep the spores relatively close to the ground.

Wilson and Baker (42) described the dispersal pattern of spores moving out from a known source as a horizontal cone having its apex at the source and its base oriented to the direction of the average wind. A section through the cone perpendicular to the center line cut out an ellipse instead of a circle, since the turbulent dispersal of spores was smaller in the vertical direction than in the horizontal direction. They stated that the density of spores (or the number of spores passing through a unit area of a plane intercepting the cone at right angles to the axis) at any distance varied approximately in inverse proportion to the square of the distance from the source ( $1/X^2$ ). Theoretical calculations compared closely with experimental evidence of their own and with that of Boevsky (16), Lambert (30), and Stepanov (39). The inverse proportion was not altered by wind velocities between 2 and 16 m.p.h.

Sutton (40) agreed with Wilson and Baker's findings except that particle diffusion varied inversely with slightly less than the square of the distance from the source ( $1/X^{2-n}$ , where  $n$  probably equalled  $1/4$ ).

Table 4 is a comparison between the theoretical values according to Wilson and Baker (42) and Sutton (40) and the actual spore counts obtained from samplers 3 ft. above the ground. Spore counts are averaged from 4 different experiments and are stated as number of spores per sampler per hour. Since the number of spores released was unknown,



Table 4. A comparison of actual spore counts obtained 3 ft. above the ground downwind from a known source of spores with theoretical estimates of spore counts for the same distances. Values are based on the number of spores caught 3 ft. in front of the source of spores and are expressed as spores/sampler/hour.

Distance from spore source (Ft.)	$1/X^2$ (Wilson & Baker, 1946)		$1/X^{7/4}$ (Sutton, 1947)		Actual Values	
	Theor. #	Theor. %	Theor. #	Theor. %	#	%
33	21.6	0.826	38.5	1.469	183.8	7.015
92	2.8	0.106	6.4	0.244	30.0	1.145
180	0.8	0.028	2.0	0.076	5.6	0.213

the number of spores caught by the sampler located 3 ft. in front of the spore source was taken as the reference value, and this was used to calculate the expected density of spores at 33, 92, and 180 ft.

At each distance from the spore source the experimental value was greater than either corresponding theoretical value. Neither diffusion coefficient,  $1/X^2$  or  $1/X^{7/4}$ , resulted in values high enough to be similar to the actual spore counts. There may be several reasons for this difference.

Previous studies of this nature made use of microscope slides coated with a sticky substance to catch spores (16, 30, 39, 42). The slides were either mounted in an upright position with the sticky face toward the spore source or placed face up on the ground. In either position a glass slide is not an efficient air sampling device and the efficiency varies with wind speed (18). The rotorod sampler used in the present study has a sampling efficiency of approximately 90 per cent, and the efficiency is independent of wind speeds up to 11 m.p.h. (1). High sampling efficiency results in spore counts more representative of the true density of spores in the air. The rotorod sampler, therefore, contributed to spore counts higher than that theoretically expected.

All of the previously mentioned studies on spore dispersal were done in open fields. Air movement in the woods is different from that in an open field (21, 29, 43, 46). In forest stands the mean wind direction remains constant for only short distances, especially if the velocity is less than 5 m.p.h. The prevailing wind is altered by cross

currents and updrafts more than it is outside the stands. Bushes and trees are barriers which change the course of the wind and slow its speed. Fungus spores riding air currents in the woods are no doubt subjected to minor changes in direction of travel due to the above factors in addition to being carried in the general direction of the prevailing wind. The cone-shaped dispersal of spores from a known source is no doubt different in the woods than in an open field, and the pattern would probably differ in shape from stand to stand depending on stand density, vegetation type, tree height, topography, and other factors. It is not surprising that results from spore dispersal studies in forest stands do not correlate with results obtained in open fields.

## THE PRESENCE OF ASCOSPORES IN AN ISOLATED ASPEN STAND

Eradication is one method that has been suggested for controlling canker diseases, including hypoxylon canker. No attempt has been made to eliminate all the infected aspen from a stand and in this way control the disease. The present study was established in Itasca State Park to evaluate the effectiveness of eradication as a control practice for hypoxylon canker.

Materials and Methods

The site chosen for the study, about 300 by 400 ft., had been cleared for use as a ball field approximately 30 years ago. The area was never used for ball and aspen, plus a few birch, invaded the cleared area. The aspen are now up to 20 ft. tall, and the surrounding stand consists of jack pine that are about 60 ft. in height. The closest hypoxylon infection north, east, south, and west of the isolated aspen stand is 1000, 1200, 400, and 660 ft. respectively.

For 3 consecutive summers concerted efforts were made to locate and remove every hypoxylon canker in the area. The fourth summer, 1963, rotorod samplers were located in the stand and the air was sampled for the presence of H. pruinatum ascospores before and after the cankers found in that year were removed. Twelve samplers were situated in such a way as to obtain a representative sample of the air in the area. One-half of the samplers were in the open and the other half under the trees. Two samplers were situated 30 ft. from the surrounding jack pine in each of the four cardinal directions, and the remaining 4 samplers were near the center of the area.

Air sampling for H. pruinatum spores was started immediately after rains which deposited enough water to wet the tree trunks. Samplers also were located in an infected aspen stand, one mile distant, to make sure ascospores were liberated after the rain.

### Results

The results of air sampling before the cankers were eradicated in 1963 are in Table 5. After this, 19 hypoxylon cankers were found and removed, and 14 of these had perithecial stromata capable of ejecting ascospores. The area surrounding the isolated aspen stand out to a distance of 400 ft. was then scouted for hypoxylon cankers, and, although there were very few aspen trees in the predominantly jack pine stand, 50 cankers were located. After all these cankers were removed the air was again sampled for hypoxylon spores after rains. The results from one of these samples (Table 6) show that just as many hypoxylon-like spores were collected after the cankers had been removed from the area as were collected before eradication. During this trial the wind was from the northwest, and the nearest known hypoxylon canker capable of spore dispersal in that direction was 660 ft.

The data in both tables show a relatively uniform deposition of spores on the study area. There were no large spore collections at the north and west sampling locations as one might expect from a "seeding out effect" as the wind, coming over the tops of the jack pine, decreased in speed.

Positive identification of the spores collected and assumed to be H. pruinatum ascospores was not determined because the adhesive

Table 5. Numbers of ascospores collected in an isolated aspen stand before hypoxylon cankers were eradicated. Rotorod samplers were started at 4:20 P.M. after a rain which deposited 2.26 inches of water. Samplers I and I' were in an infected aspen stand one mile away.

Sampling location	Hours								Total spores	
	1	2	3	4	5	6	7	8		
N Edge A	3	6	0	4	6	9	2	3	33	
	A'	1	6	2	3	0	4	1	1	18
E Edge B	6	5	4	3	3	7	0	0	28	
	B'	1	1	0	4	6	7	1	0	20
S Edge C	3	13	10	7	6	6	0	2	47	
	C'	3	6	0	0	5	1	1	1	17
W Edge D	2	3	3	0	3	7	3	0	21	
	D'	0	0	1	6	1	4	0	0	12
Center E	0	4	3	5	2	3	4	0	21	
	F	1	12	1	9	2	2	0	0	27
	G	3	4	2	2	0	2	0	0	13
	H	0	0	0	1	4	4	2	0	11
Totals	23	60	26	44	38	56	14	7	268	
Infected stand	I	0	583	1684	9600	3000	2040	193	68	17,168
	I'	0	225	233	418	64	90	14	1	1,045

Table 6. Numbers of ascospores collected in an isolated aspen stand after the stand had been surveyed for hypoxylon canker and all the cankers had been removed. Rotorod samplers were started at 7:30 P.M. after a rain which deposited 0.37 inches of water. Wind was from the northwest at less than 5 m.p.h. Samplers I and I' were located in an infected aspen stand one mile away.

Sampling location	Hours					Total spores
	1	2	3	4	5	
N Edge A	7	6	5	2	1	21
A'	9	6	3	0	0	18
E Edge B	6	7	1	2	0	16
B'	7	5	4	0	0	16
S Edge C	13	7	10	1	0	31
C'	4	12	9	1	0	26
W Edge D	2	5	7	1	0	15
D'	14	4	8	1	0	27
Center E	8	5	2	0	0	15
F	-	6	5	0	1	12
G	9	23	1	0	0	33
H	7	10	7	0	0	24
Totals	86	96	62	8	2	254
Infected I	486	849	8	2	406	1,751
stand I'	206	210	20	1	-	437

on the collector bars of the rotorod samplers was toxic to the spores, thus prohibiting culturing. Culture plates were not exposed to the air, and it is very unlikely that any spores would have been collected using such a technique unless an unreasonably large number of exposures were made. The spores collected by the samplers resembled those of H. pruinatum in size, shape, and color. However, other species of Hypoxylon in the area could have been ejecting spores.

#### Discussion

Results of this study indicate that at least during a period of 4 years it was virtually impossible to completely eliminate hypoxylon canker from an isolated aspen stand. After 3 years of eradication, the fourth survey located 19 additional cankers. Perithecial stromata on 14 of these cankers indicated that these had been present in previous years in spite of the thoroughness of the surveys. Normally stromata are not formed on a canker until the canker is at least 3 years old.

Rotorod samplers, located in this isolated aspen stand, from which supposedly all the hypoxylon cankers had been removed, collected hypoxylon-like spores from overlooked cankers or from cankers outside the stand. If coming from outside, the spores were collected by the samplers at the rate of 51 spores per hour. The closest known cankers were located 660 ft. upwind. During more optimum conditions of spore ejection and wind dissemination, larger spore showers could be expected on the stand. There is little doubt that new infections in the stand could originate from inoculum produced in another area and carried into the stand by wind.



## DETERIORATION OF HYPOXYLON CANKERS ON STANDING AND FELLED TREES

A method commonly mentioned for controlling forest tree diseases is to fell and remove the infected trees. This practice can be carried out in an improvement cut or during periodic thinning operations. Such a control measure, in some cases, results in the slowing down or arresting of disease spread as a result of a reduction in inoculum produced due to the death and desiccation of the host. No work has yet been reported evaluating this control for hypoxylon canker.

A study plot was established in northern Minnesota in which the deterioration of cankers, caused by H. pruinatum, was compared for cankers on trees lying on the ground and cankers on standing trees. The Itasca Park area, where this plot was located, is within the natural range of aspen and the stand was typical of many aspen stands throughout the Lake States region.

### Materials and Methods

The 21 trees with cankers used in the study represented all the standing aspen in the plot which had cankers producing perithecial stromata. Of these cankered trees, some appeared in good health, some in poor health based on dying leaves and branches, and other trees were dead. The trees were numbered, marked with paint, and tree diameter (d.b.h.) and health, stromatal production on the cankers, and other information pertinent to the study were recorded.

Twenty-one additional hypoxylon-infected trees outside the plot were felled and the cankered sections removed. Data similar to those obtained for the standing trees were recorded for these trees and

cankers. The cankers were brought into the plot and placed on the ground beside the standing cankered trees, the condition of each canker on the ground matching as closely as possible the canker on the standing tree beside it (Fig. 13).

Vaseline-coated microscope slides were mounted by means of paper clips over several stromata on each canker for collecting ascospores. Up to six slides were mounted on each tree. White paint was used to outline the perimeters of the areas producing stromata (Fig. 14). This facilitated the detection of new stromata later in the study.

Periodically throughout the year slides were checked for ascospore discharge and cankers were examined for new stromatal production. New slides were then exposed and new stromata encircled with paint.

## Results

Data on the cankers were recorded for 26 months, and the information on ascospore ejection during this period is in Table 7, that on new stromatal production in Table 8. The same data are presented graphically in Figures 15 and 16.

Spore production was significantly reduced by felling cankered trees but not until after the cankers were on the ground for 23 months. Up to 23 months, there was a steady decrease in numbers of cankers ejecting spores, with a small increase after the winter months (Fig. 15, 20 months). At the end of the study all of the cankers on standing trees were still ejecting spores, while only 24 per cent of the felled cankers were ejecting spores (Fig. 15). Three standing trees died



Figure 13. Hypoxylon cankers on a standing and a felled tree. Both cankers were in a similar stage of development at the start of the study. White paint marks the areas where stromata were present.



Figure 14. A microscope slide mounted on a canker to collect ascospores of H. pruinatum ejected from stromata. White paint marks the area within which stromata are present. The canker extends beyond the limits of the photograph.

Table 7. Ascospore ejection, as measured by deposits on microscope slides mounted over perithecial stromata, from cankers on standing and felled trees.

Standing Cankers									Felled Cankers								
Tree vigor	Tree d.b.h.	Months							Tree vigor	Tree d.b.h.	Months						
		8	10	11	14	20	23	26			8	10	11	14	20	23	26
*D '61	3 in.	+	+	+	+	+	+	+	D '61	3 in.	+	+	+	-	+	-	-
Good	2 1/2	+	+	+	-	+	+	+	Poor	2	+	+	+	+	+	-	-
D '58	2	+	+	+	+	+	+	+	D '58	5	+	+	+	+	+	-	-
D '59	1 1/2	+	+	+	+	+	+	+	D '59	2	+	+	+	-	-	-	-
Poor	2	+	+	+	+	+	+	+	Good	3	+	+	+	+	+	-	+
D '58	1 1/2	+	+	+	+	+	+	+	D '58	4	+	+	+	+	+	-	-
Poor	4	+	+	+	+	+	+	+	Good	4	+	+	+	+	+	-	-
Poor	2 1/2	+	+	+	+	+	+	+	Good	4	+	+	+	+	+	+	+
Good	2 1/2	+	+	+	+	+	+	+	Good	4	+	+	+	+	+	-	-
Good	2	+	+	+	+	+	+	+	Good	5	+	+	+	-	-	-	-
D '58	1 1/2	+	+	+	+	+	+	+	D '58	2	+	+	+	+	+	+	+
Good	1 1/2	+	+	+	+	+	+	+	Good	2	+	+	-	-	-	-	-
D '59	2	+	+	+	+	+	+	+	D '59	2	+	+	+	-	-	-	-
Good	3	-	+	+	+	+	+	+	Good	2	+	+	-	-	-	-	-
Good	2	+	+	+	+	+	+	+	Good	2	+	+	-	-	+	-	-
Good	4			+	+	+	+	+	Good	3	+	+	+	+	+	+	-
Good	3	+	+	+	+	+	+	+	Good	4	+	+	+	-	+	+	-
Poor	2	-	-	+	+	+	+	+	Good	4	+	+	+	-	+	+	-
Poor	3	+	+	+	+	+	+	+	Good	3	+	+	+	+	+	+	-
D '59	3	+	+	+	+	+	+	+	Poor	1 1/2	+	+	+	+	+	+	+
Good	2 1/2	+	+	+	+	+	+	+	D '59	4	+	+	+	+	+	-	+
Good	2 1/2	+	+	+	+	+	+	+	Good	1 1/2	+	+	+	+	+	+	-
Totals	21	18	19	21	20	21	21	21	Totals	21	21	21	18	12	16	7	5

\* D= Estimated death in 1961.

+ = Spores present; - = spores absent.

Table 8. New stromata production over a period of 26 months on standing and felled cankers.

Standing Cankers								Felled Cankers							
Tree vigor	Tree d.b.h.	Months						Tree vigor	Tree d.b.h.	Months					
		8	10	14	20	23	26			8	10	14	20	23	26
*D '61	3 in.	+	+	+	-	+	-	D '61	3 in.		-	-	-	-	-
Good	2 1/2		-	+	+	+	+	Poor	2		-	-	-	-	-
D '58	2		+	+	+	-	-	D '58	5		-	-	+	-	-
D '59	1 1/2		+	+	-	+	-	D '59	2		-	-	-	-	+
Poor	2		+	+	+	-	-	Good	3		+	-	-	+	-
D '58	1 1/2		+	+	+	-	+	D '58	4		-	-	-	-	-
Poor	4		+	+	+	+	+	Good	4		+	+	-	-	-
Poor	2 1/2		+	+	-	+	-	Good	4		+	+	-	-	-
Good	2 1/2		+	-	+	-	-	Good	4		+	+	-	-	-
Good	2		+	-	+	-	+	Good	5		-	-	-	-	-
D '58	1 1/2		+	+	+	-	-	D '58	2			+	-	-	-
Good	1 1/2	+	+	+	+	+	+	Good	2	+	-	-	-	-	-
D '59	2	-	+	+	-	-	-	D '59	2	+	-	-	-	-	-
Good	3	-	+	+	-	+	-	Good	2	-	-	-	-	-	-
Good	2	-	+	+	+	+	+	Good	2	+	-	-	+	-	-
Good	4	+	+	+	-	+	+	Good	3	-	-	-	+	-	-
Good	3	-	+	+	+	+	+	Good	4	-	-	-	-	-	-
Poor	2	-	+	+	+	+	+	Good	3	-	-	-	-	-	-
Poor	3	-	+	+	+	+	+	Poor	1 1/2	-	-	-	-	+	+
D '59	3	+	+	+	-	+	+	D '59	4	-	+	-	+	-	+
Good	2 1/2	+	+	+	-	+	-	Good	1 1/2	-	-	-	-	-	-
Totals	21	+	20	19	13	14	11	Totals	21	+	6	3	4	2	3

\* D = Estimated death in 1961; + = New stromata present; - = New stromata not present.

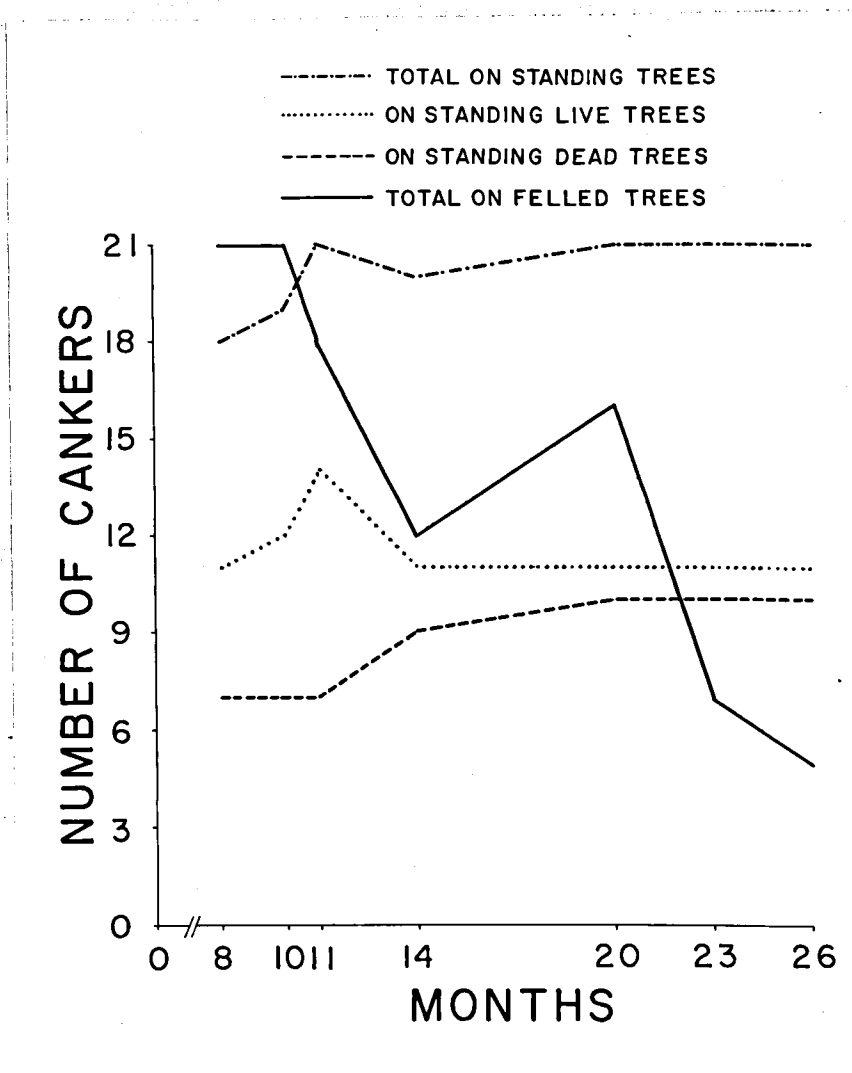


Figure 15. The number of hypoxylon cankers that ejected ascospores at various intervals in a 26-month period. The study was started in Sept., 1961.

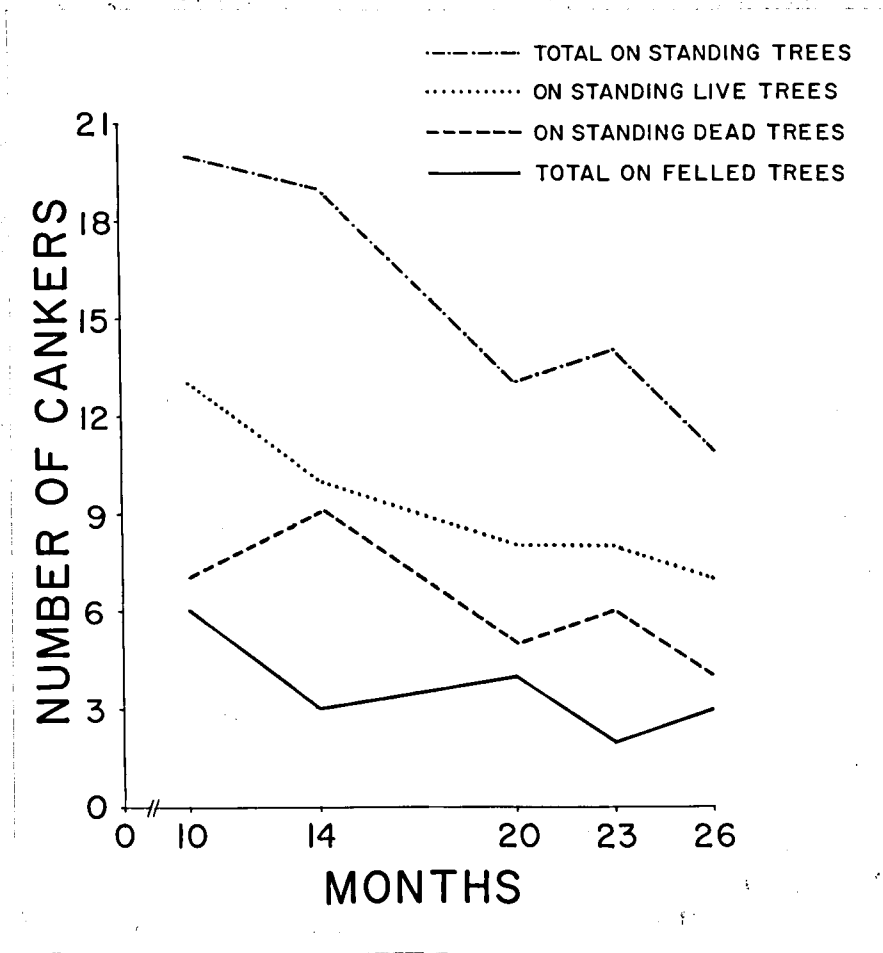


Figure 16. The number of hypoxylon cankers on which new stromata were produced during a 26-month period. The study was started in Sept., 1961.



during the study, and after death, data from these trees were tallied with those of the other dead trees. There was no apparent correlation between tree size or health and duration of spore production on the felled or standing cankers.

All the cankers had stromata at the start of the study. Felling aspen with cankers immediately reduced new stromatal production (Fig. 16). After 10 months, 95 per cent of the standing cankers produced new stromata while only 29 percent of the felled cankers produced new stromata. Stromatal production decreased throughout the remainder of this study on both standing and felled cankered trees. There was no difference in rate of decrease between live and dead standing trees.

On felled cankers, stromata sometimes appeared outside the limits of the original canker. Mycelium of the pathogen had ramified throughout the bark of the trunk section without producing the mottled symptom typical of diseased bark. Apparently this symptom does not occur when the fungus invades dead tissues.

As the number of felled cankers producing spores decreased, spore deposition increased on the slides placed over stromata which were ejecting spores. Slides on standing cankers sometimes were covered with the black ascospores, but even more often spores were deposited in such large quantities on slides on felled cankers that the deposits were raised above the slide surface. Cankers on the ground seemed to eject more spores per stromata in a given time than did standing cankers. The reason for the great abundance of spores on these slides was probably due to more available moisture near the

ground and more frequent and longer periods of spore ejection.

### Discussion

The data indicate that a canker on a standing tree can eject spores for at least 26 months whether the tree is alive or dead. Even standing trees which have been dead for 5 years are capable of liberating inoculum into the air (Table 7).

Although ascospore ejection was reduced after 23 months when cankered trees were felled, the amount of inoculum liberated into the air before this time may have been greater than that liberated from cankers in standing trees. No attempt was made to germinate the spores, but assuming the spore germination percentage to be the same from standing and felled cankers, significant reduction of viable inoculum in a given stand would not be achieved by felling hypoxylon-infected trees until nearly two years after the trees had been felled.

## ASCOSPORE GERMINATION STUDIES

Most attempts to inoculate aspen with ascospores of H. pruinatum have not resulted in canker formation. The ability of ascospores to germinate and penetrate host tissue must be dependent on a combination of environmental factors and on certain host-parasite characteristics which do not commonly occur simultaneously. To determine what some of these factors are optimum temperature and pH for spore germination on an agar medium was determined, as well as the effects of the various layers of aspen bark on spore germination.

Materials and Methods

Ascospores were obtained from perithecial stromata collected in several aspen stands 30 miles north of Minneapolis. The stromata were soaked for 2 hours in water containing 100 p.p.m. neomycin or albamycin, then placed in petri dishes. The antibiotic treatment was needed because the stromata were frequently contaminated with bacteria and the bacteria inhibited spore germination. Ejected ascospores were collected on glass slides mounted over the stromata. The slides were stored in sterile slide boxes until used.

The medium used for the pH study was 2 per cent water agar buffered with the phthalate-sodium hydroxide buffer system in the low pH range and with the phosphate buffer system in the high pH range (17). The medium for the temperature study was water agar adjusted to pH 6.0. All media were amended with 100 p.p.m. antibiotic to inhibit bacterial growth. Both buffers and antibiotics were added aseptically to the autoclaved agar when the latter had cooled to 45° C.

Aspen bark, collected from trees about 2 inches in diameter, was surface sterilized with 70 per cent alcohol. The bark was sectioned tangentially into wafers, 15 to 30 microns thick and 4 to 25 mm sq., with a razor blade and hand microtome. Three separate bark layers were used in the study, including the outer thin layer (phellem), the soft green layer (phelloderm and cortex), and the secondary phloem. Wafers also were sectioned from the outer two annual rings of sapwood. One wafer from each of the 3 bark layers and one wafer of sapwood was placed on each glass slide. Slides were placed in 8-ounce jars containing water to maintain a nearly saturated relative humidity.

Spore suspensions were prepared containing from 250,000 to 400,000 spores per ml of sterile distilled water containing 100 p.p.m. antibiotic. Approximately one ml of spore suspension was spread on each agar plate and 2 to 3 drops of suspension placed on each of the 4 wafers on the slides.

Petri dishes in the pH study were incubated at 27°C while the jars containing bark layers were placed at 23°C. Percentage spore germination, after 72 hours, was based on the total number of spores in 5 fields at 100X magnification on each plate and 3 fields at the same magnification on each bark and wood wafer.

### Results

Ascospores germinated in media from pH 4.4 to 6.7, and germination was less than 5 per cent at these extremes. Figure 17 shows spore germination in the pH range from 5.0 to 6.7. Optimum germination was at pH 5.7. Where the buffer systems overlapped between pH 5.8 and 6.3 there were no significant differences in spore

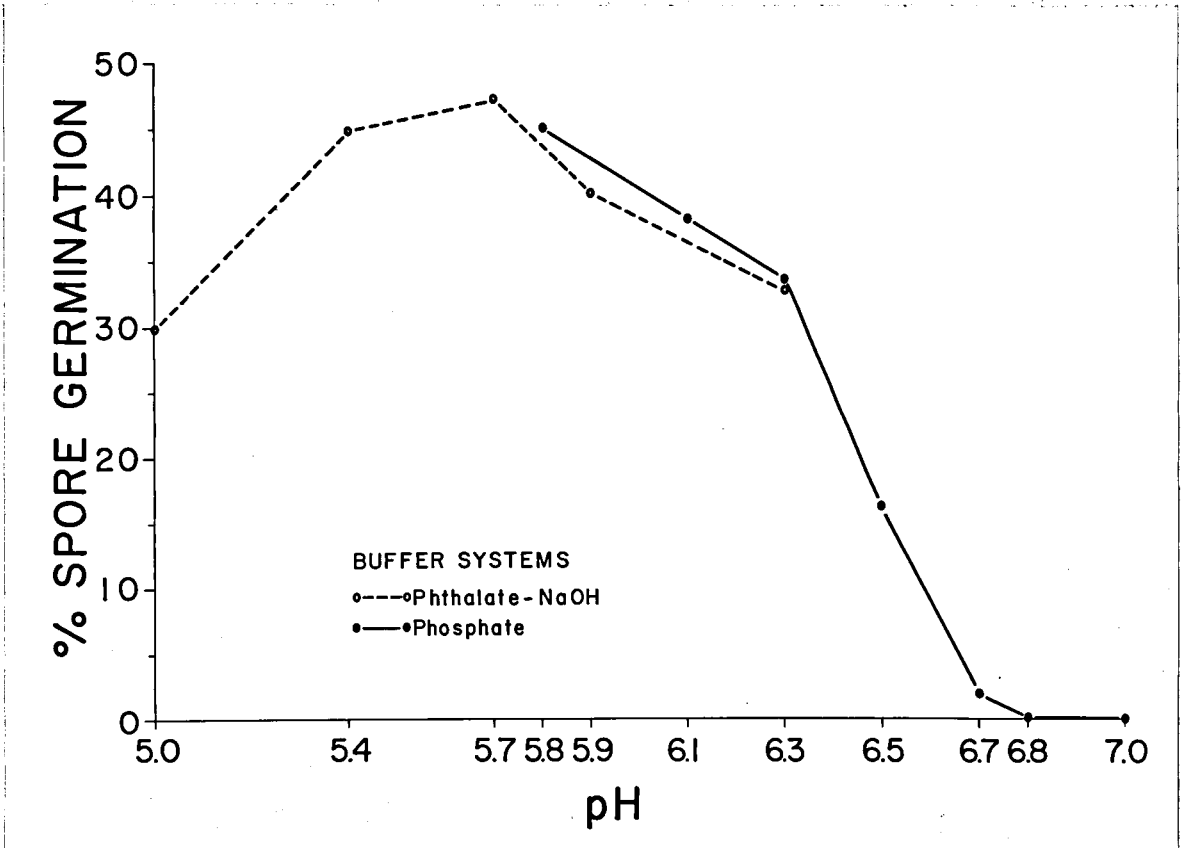


Figure 17. Percentage spore germination at a range of pH from 5.0 to 7.0. The medium was amended with 100 p.p.m. albamycin to inhibit bacterial growth. Percentage germination was based on the number of spores in 5 fields at 100X magnification. There were 6 replicates.

germination for the two buffers. Spore germination was the same whether the media were amended with neomycin or albamycin.

Figure 18 shows the percentage spore germination from 20 to 35° D. No germination occurred at or below 15° C nor at or above 39° C. Optimum temperature for spore germination in this test was 31° C; in another test run in the same manner it was 30° C.

The results of spore germination on bark and wood layers are in Figure 19. The green phelloderm layer almost completely inhibited ascospore germination. After 72 hours, only 3 spores out of 584 had germinated. With the same spore suspension, percentage germination on the phellem layer, 15 per cent, and on the secondary phellem, 13 per cent, was similar to germination on water agar. Percentage spore germination on the sapwood, 44 per cent, was three times that on any bark layer or on water agar. In the free water on the slides around both the sapwood and secondary phloem wafers spores were germinating. Fewer spores germinated in the water about phellem wafers and none in the water around phelloderm wafers. Spore germination percentages that occurred in the free water were not recorded.

### Discussion

An optimum pH of 5.7 for ascospore germination was similar to the value reported by Gruenhagen (25) for maximum mycelial growth of the fungus in liquid culture. However, the pH range in which ascospores germinated was narrower than the range that allowed mycelial growth. Gruenhagen reported that mycelial growth occurred between pH 3.0 and 8.0. Ascospores did not germinate below pH 4.4 nor above pH 6.7.

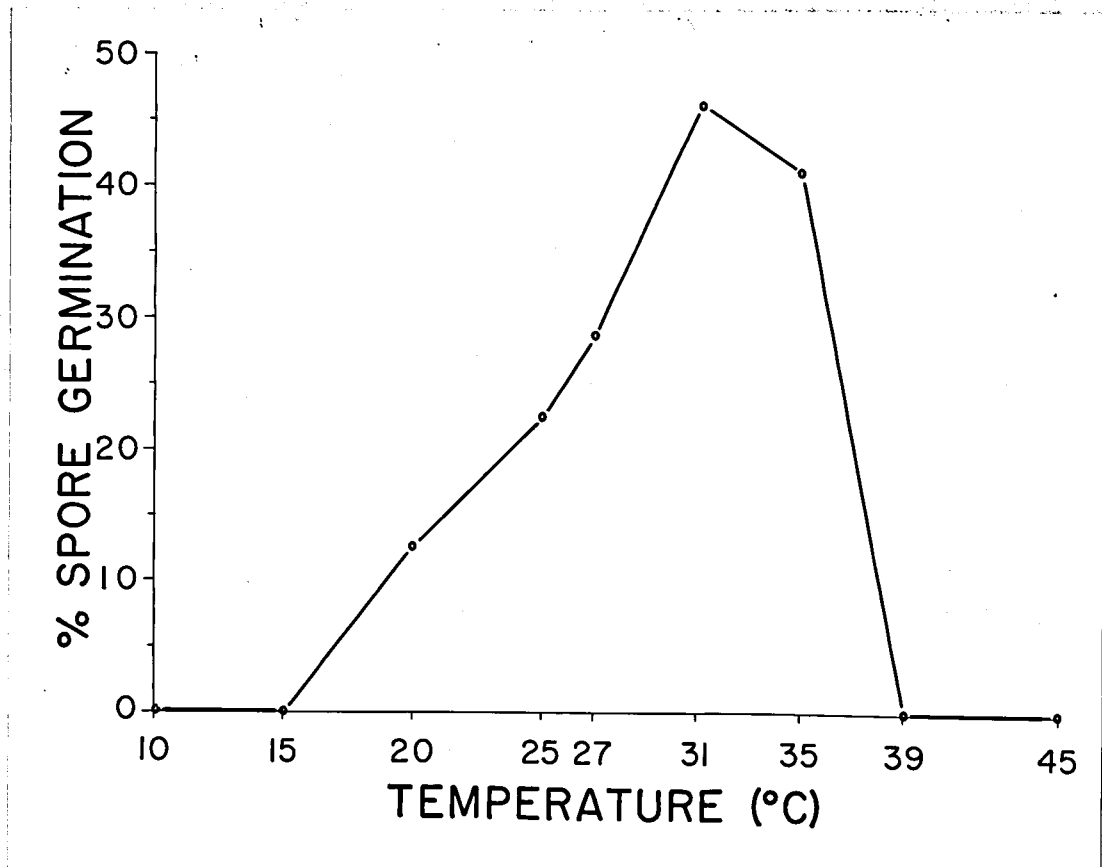


Figure 18. Percentage spore germination at a range of temperatures from 10 to 45°C. The medium was buffered to pH 6.0 and amended with 100 p.p.m. albamycin to inhibit bacterial growth. Percentage germination was based on the number of spores in 5 fields at 100X magnification. There were 6 replicates.

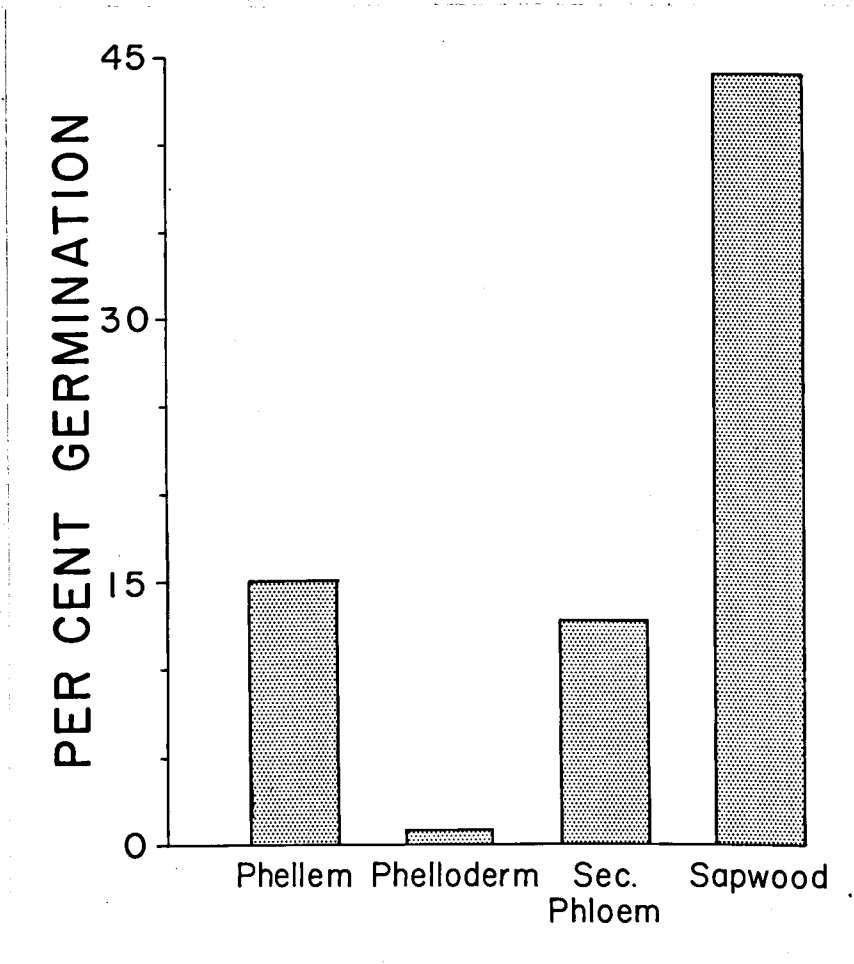


Figure 19. Percentage spore germination on 3 different layers of aspen bark and on sapwood. Incubation was at 23°C, and the percentage germination was based on the number of spores in 3 fields at 100X magnification. There were 6 replicates.



Maximum spore germination, on water agar at pH 6.0, occurred at 30° or 31°C. Oshima (35) found that maximum spore germination was at 25°C in drops of water containing small squares of phellem tissue. He also reported only 5.5 and 5.0 per cent germination at 20° and 30°C respectively. Gruenhagen (25) reported maximum mycelial growth at 28°C with a range between 12° and 32°C. In the present study the range within which spores germinated was of about the same magnitude as the range for mycelial growth, except that the minimum and maximum temperatures were both increased 3 to 8 degrees.

Sapwood tissue stimulated the germination of ascospores when compared to spore germination on water agar medium at 23°C. French and Oshima (22) reported that a thin square of phellem tissue in a drop of water stimulated ascospore germination, whereas secondary phloem tissue had no more effect on germination than did the distilled water control. In the present study neither phellem nor secondary phloem tissue stimulated or inhibited spore germination when compared with germination on water agar medium. French and Oshima further reported that spore germination was almost completely inhibited by the presence of phelloderm tissue. Similar results were recorded in the present study. The high percentage germination of ascospores on sapwood, 3 times that on any bark layer, suggests that open wounds may be the best infection locations in nature.

French and Oshima (22) noted that the phelloderm layer was, in most cases, absent from the bark immediately above a branch axil. Surveys in Minnesota indicate that the majority of infections can be traced back to branch axils. Artificial inoculations with ascospores, including pregerminated spores, at these points have not resulted in

cankers. Perhaps this was due to the absence of other conditions necessary for successful colonization of host tissue.

Three chemicals found in aspen bark have been reported to inhibit the growth of H. pruinatum in culture (27, 28). These compounds include pyrocatechol, salicylic acid, and an unidentified glycoside. The fungistatic chemicals have been isolated in high concentrations from all parts of the trunk except from the upper stem, and this is where most cankers are found. It would be interesting to know if the compounds are located in a particular portion of the bark and if this site might be the inhibitory green phelloderm layer.

Knowledge of the conditions necessary for spore germination represents only a portion of the knowledge needed for full understanding of the disease cycle initiated by H. pruinatum. Hyphal penetration of host tissue may be dependent on a completely different set of factors than those necessary for optimum spore germination. It may be that in nature H. pruinatum spores germinate and the host is colonized under suboptimal conditions. Further work is needed to elucidate the infection phenomenon.

## ASPEN BARK MOISTURE CONTENT DURING THE DORMANT SEASON

Bier (8, 9, 10, 11, 12, 13) supports the idea that the susceptibility and resistance of certain tree species to attack by facultative parasites is dependent, in part, on the amount of water in the bark of the tree hosts, i. e. bark turgidity percentages, as determined by the following formula:

$$\frac{\text{weight of water in bark sample} \times 100}{\text{weight of water required to saturate the same sample}}$$

Bier found that the threshold level of bark moisture for the growth of H. pruinatum in aspen, expressed as relative turgidity percentate, was approximately 75 to 77 per cent. Less than 75 per cent relative turgidity in the bark rendered aspen susceptible to ingression and invasion by the pathogen. When placed in a beaker of water, aspen stem cuttings, infected with H. pruinatum and with bark relative turgidity percentages less than 75 per cent, increased in bark moisture to above 80 per cent relative turgidity and the growth of the fungus in the stems was arrested (13).

Bier further reports (13) that the bark moisture content of aspen fluctuates throughout the year. In July, the relative turgidity percentage is in the 90's, while during the dormant season, i. e. December, bark relative turgidity is 80 per cent or below. From these findings, and from the report by Wood and French (45) that ascospores of H. pruinatum are ejected during the winter, Bier (14) suggested that aspen are most susceptible to infection by H. pruinatum during the dormant season when the bark moisture content is below the threshold level for the pathogen's growth in the bark and that perhaps infection

in nature takes place during this period.

To determine aspen bark moisture contents during the dormant season in Minnesota, plots were established in aspen stands at the Cedar Creek Natural History Area in which percentage relative turgidity of aspen bark was determined monthly during the dormant season of 1962-63 and part of the dormant season of 1963-64. The dormant season was designated as the period between leaf fall and bud swelling, mid-October to mid-April respectively.

#### Materials and Methods

Four aspen stands were selected for the study; 2 of the stands had hypoxylon cankers present and 2 were infection-free. Of the 2 infected stands, one contained only aspen while the other had additional deciduous species. Anderson (2) found that the presence of other tree species was associated with a higher incidence of hypoxylon infection. Of the 2 infection-free stands, one contained only aspen while the second stand had other species of trees intermixed with the aspen. All 4 study plots had at least one side exposed to an open field. Anderson (3) reported that "Within a given stand about 2 to 3 times as many infections occur on trees near exposed stand edges as occur on trees within uniformly well-stocked parts of the stand."

Fifteen non-infected aspen, from 1 to 4 inches d.b.h., in each plot were selected at random as the test trees. Bark moistures were determined 6 times for each tree during the dormant season. The bark samples, from an area of the trunk between 4 and 6 feet above the ground, were obtained with a 2 cm. diameter cork borer. Approximately 1 gm. of fresh bark tissue was taken for each moisture determination.

Care was taken to avoid bark which was obviously water soaked from recent rains or which had been exposed to direct sunlight for several hours. Fresh bark samples were placed immediately in tightly sealed metal cans and within 30 minutes original weights were determined to the nearest thousandth of a gram. The bark samples were then placed in water for 24 hours, saturated weights were recorded, and the samples were dried at 90°C for 6 hours and weighed a final time. Bark relative turgidity percentages were ascertained according to the above mentioned formula. This procedure of determining bark moisture content was more accurate for comparative studies than was determination based only on oven dry weight in that the percentage relative turgidity method discounted variability in bark density.

During the last week of March, 1963, the bark moisture contents of 3 aspen, 1, 2, and 3 inches d.b.h., were determined for 3 consecutive days to check for differences in moisture contents between the trees and for differences resulting from increasing time since the previous rain (sampling started 2 days after a rain). Bark samples were obtained from 4 locations on the main stem of the same 3 trees, 1, 3, 5, and 7 feet above the ground. Bark samples also were taken from 3 branches on each tree at 0, 1, and 2 feet from the trunk. The lowest 3 branches on each tree were sampled the first day and the next higher two groups of 3 branches each on the following 2 days. All bark samples were obtained between 9 and 11 A.M. each day and original weights determined within 30 minutes of sampling.

## Results

The average relative turgidity percentages of aspen bark from each of the 4 plots remained between 80 and 90 per cent from October 25 to April 27 (Fig. 20). In October and November, 1962, averages in all 4 plots were between 80 and 83 per cent, the lowest moisture contents for the 1962-63 dormant season. Average bark moisture contents from December through April were, on the whole, above 83 per cent. Throughout the dormant season the average bark moisture content of aspen in the 2 diseased plots tended to be less than that in the 2 infection-free plots, but the differences were not significant. No significant differences were noted in average bark moisture contents between aspen stands with and without other species. At no time during the dormant season did the average relative turgidity in any plot drop as low as the moisture threshold level for hypoxylon growth in aspen bark (75-77 per cent).

Bark moisture content determinations in Oct., Nov., and Jan. of the 1963-64 dormant season indicated that average relative turgidity percentages in all 4 aspen stands remained between 81 and 86 per cent. The average bark moisture content of trees in the hypoxylon infected mixed aspen-hardwood stand was always between 2 and 4 percentage points below the averages for trees in the 2 noninfected stands, but the differences were not significant. Values from the hypoxylon infected pure aspen stand were always similar to those from the 2 noninfected stands.

Bark moisture contents were variable within individual stands, the range of relative turgidities ranging over 22 percentage points in

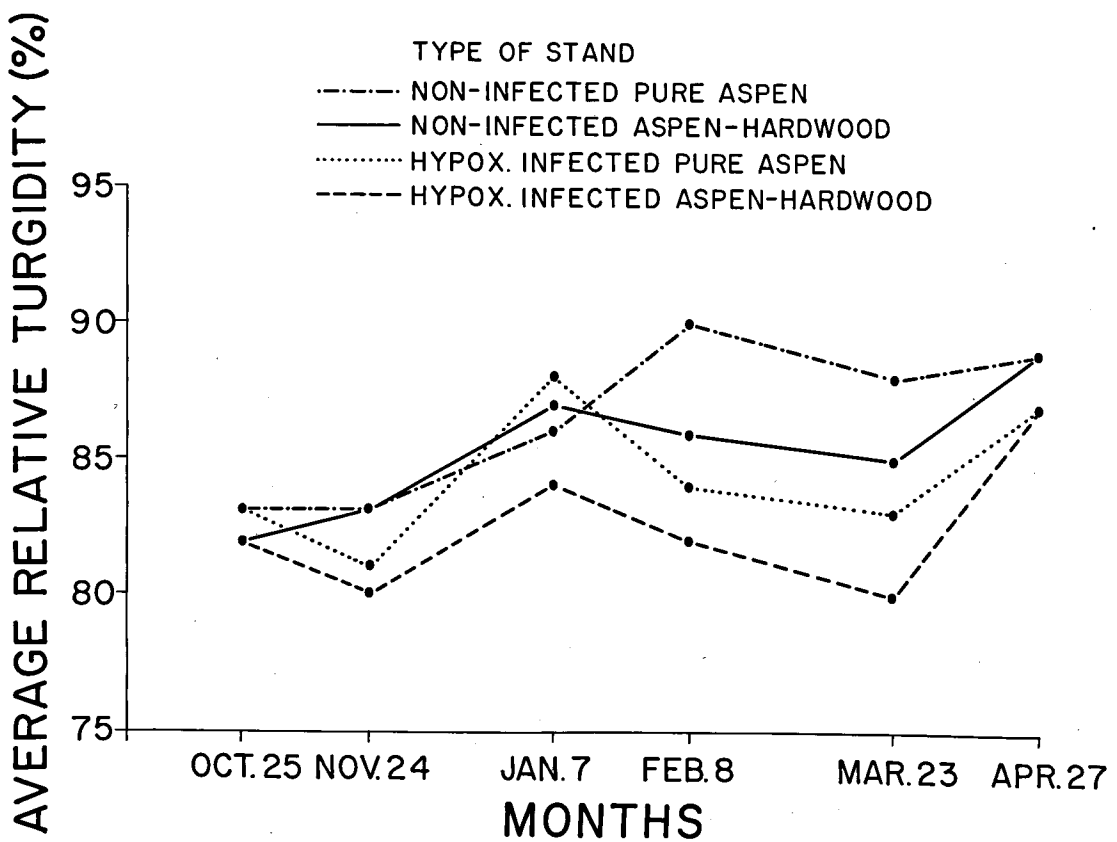


Figure 20. Average relative turgidities of aspen bark in 4 stands during the dormant season of 1962-63. Leaf fall was before Oct. 25, and the trees were breaking dormancy on April 27.

one stand on Jan. 7 (Fig. 21). Individual trees in all stands, except the pure, infection-free aspen stand, on occasion, reached or dropped below the moisture threshold level for hypoxylon growth in the bark. This phenomenon was consistently evident in the 2 hypoxylon infected stands but was not specific for individual trees within these stands.

Average bark moisture contents from various locations on aspen 1, 2, and 3 inches d.b.h., determined on March 26, 27, and 28, 1963, did not differ significantly from tree to tree (Table 9). Neither were there significant changes in bark moisture contents during the 3 day period at specific locations on individual trees.

When bark from various locations on aspen was sampled for moisture content, percentage relative turgidity varied according to location on the tree (Table 9). In the case of the 1 inch tree, percentage relative turgidity decreased in the main stem with increased distance from the ground. This was not true in the 2 and 3 inch trees. Generally speaking, for all branches, percentage relative turgidity was highest near the trunks of the trees and lowest 2 feet from the trunks, which was as far as sampling went. The latter location usually had an average moisture content at or below the moisture threshold level for hypoxylon growth in aspen bark.

### Discussion

Bier (13) indicated that aspen susceptible to H. pruinatum attack may be identified by determining the moisture content of the bark (percentage relative turgidity). Susceptible trees were those



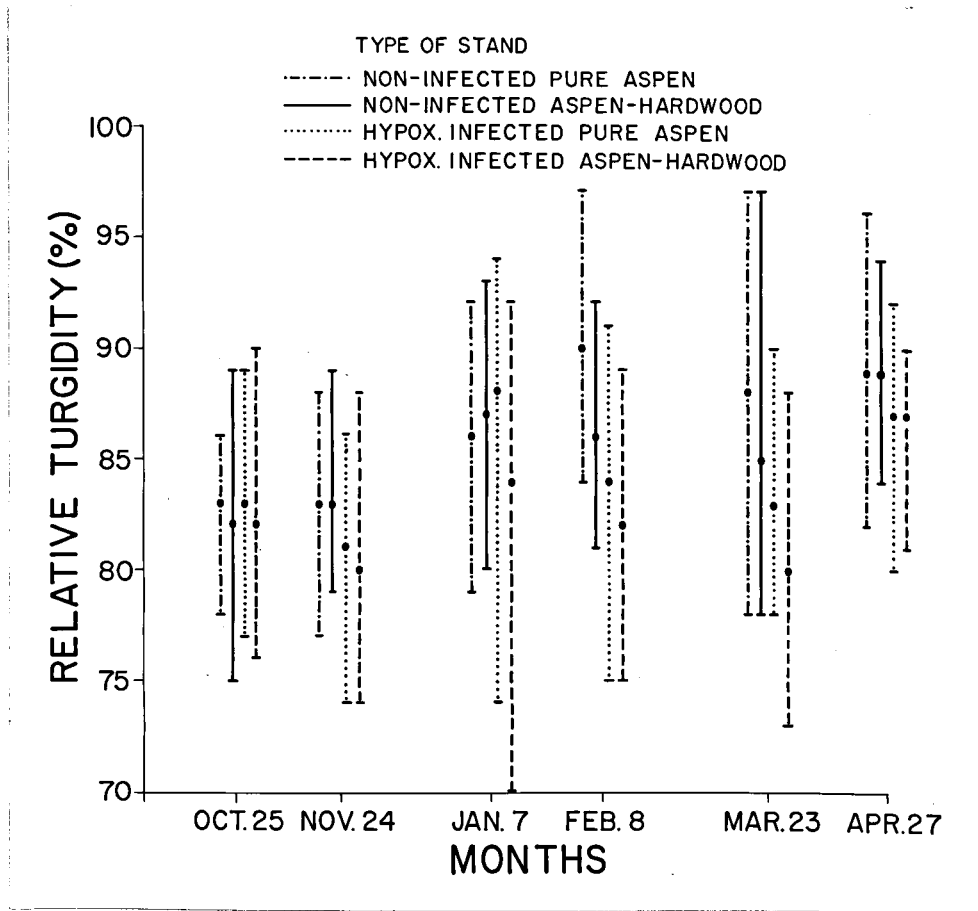


Figure 21. Ranges of bark relative turgidities in 4 aspen stands at various times during the dormant season of 1962-63. Average relative turgidities are indicated by dots.

Sampling location on tree	Ht. from ground or dist. from trunk (ft.)	Tree diameter in inches (d.b.h.)											
		1				2				3			
		3/26	3/27	3/28	Ave.	3/26	3/27	3/28	Ave.	3/26	3/27	3/28	Ave.
Trunk	1	88%	86%	86%	87%	91%	86%	82%	86%	82%	80%	86%	83%
	3	85	82	87	85	88	83	85	85	90	88	88	89
	5	82	82	83	82	88	86	86	87	86	86	80	84
	7	75	75	74	75	90	86	87	88	88	85	88	87
Lower 3 branches	0	77	72	70	73	88	84	82	85	86	83	78	82
	1	75	76	80	77	82	76	76	78	66	74	78	73
	2					76	77	78	77	76	76	76	76
Branches 4 - 6	0	80	78	75	78	79	80	79	79	86	82	84	84
	1	76	72	74	74	84	78	74	79	77	80	76	78
	2					80	72		76	75	79	72	75
Branches 7 - 9	0	74	74	70	73	78	78	77	78	80	79	78	79
	1	70	70	70	70	86	74	68	76	75	79	64	73
	2					84	76		80	76	74		75

Table 9. Percentage relative turgidities of aspen bark at various locations on 3 trees determined on 3 consecutive days in March, 1963

with bark moisture contents below 75-77 per cent, and this level might be attained by individual trees during the dormant season. Bier reported that percentage relative turgidity during the growing season was in the 90's, and this was verified during the summer of 1963 in Itasca State Park.

In the present study the bark moisture content was determined during the dormant season for healthy aspen in stands infected with hypoxylon cankers and in infection-free stands. No significant difference was recorded in bark moisture contents from the two types of stands and at no time were the average moisture contents for the stands as a whole below 80 per cent, indicating that a stand was not susceptible to attack by H. pruinatum because the average bark moisture content in the stand was above the moisture threshold level for pathogen growth in the bark.

These results did not exclude the possibility that individual aspen may, at times, have bark moisture below the threshold level. Actual data showed that a few aspen in 3 of the 4 study plots did, on occasion, have bark moisture contents at or below 75 per cent relative turgidity (Fig. 21). This situation occurred most often in the 2 stands with hypoxylon cankers, on 4 out of 6 sampling occasions for one stand and 3 out of 6 times for the other stand during the 1962-63 dormant season.

Although individual aspen may be most susceptible to attack during the dormant season, and although ascospores of H. pruinatum may be ejected from perithecia during the winter (45), the dormant

season does not appear to be a likely time for infection to occur. Previously mentioned studies showed that the minimum temperature for ascospore germination was 20°C and that the optimum temperature was 30-31°C. Ascospores might be ejected and land on susceptible hosts when the ambient temperature was only 2-3°C, but at least 36 hours of temperatures above 20°C would be necessary for spore germination.

Bloomberg (15) stated that the bark moisture content of black cottonwood (Populus trichocarpa Torr. and Gray.) was at its yearly minimum most often in October, but occasionally in December. The present study showed that the minimum average moisture content of aspen bark in all 4 study plots on any one date was in October or November. Because of the large variability of moisture contents within individual stands throughout the dormant season, these values in the fall were not proven statistically as minimum values. If, however, minimum yearly bark moisture levels were attained in October, infection by H. pruinatum is possible because ambient temperature can be favorable at this time for ascospore germination.

The bark moisture content in aspen branches, determined in the last week of March before the trees were breaking dormancy, was often low enough for H. pruinatum growth in the bark, especially at locations 2 feet out from the trunk. This might indicate that branches are more susceptible to hypoxylon attack due to low moisture levels than are the trunks.

The results presented in this study represent the bark moisture contents of aspen during only two dormant seasons. The minimum moisture levels which aspen bark reached during the dormant season,

may, in other years, be lower or higher than those attained during these two years. If Bier is correct in assuming that aspen bark below 75-77 per cent moisture content is susceptible to hypoxylon attack, then individual aspen, or entire stands, may be more susceptible to attack in some years than in others. Anderson (4) has already stated that the number of new infections fluctuates substantially from year to year and that certain years have been more favorable for infection than other years.

## SUMMARY

In this study information was collected on the factors involved in ascospore dispersal and the conditions necessary for ~~asce-~~ ~~spore~~ infection of aspen by <sup>ascospores of</sup> H. pruinatum.

When hypoxylon cankers were moistened with water to simulate rains, ascospore ejection was initiated within 1 to 4 hours, depending on the moisture condition of the cankers before adding the water, and ejection continued for periods up to 18 hours. A decrease in spore ejection, as determined by the number of spores collected by a rotorod sampler, was always correlated with an increase in ambient temperature and a decrease in relative humidity.

Spore ejection took place after the cankers had been wet for a period of time. Water condensation from a fog was enough to initiate ejection.

The density of black spore deposits on glass slides placed over individual perithecial stromata on a canker served as a representative sample of the intensity of spore ejection from all the stromata on that canker. Often individual perithecia ejected spores for the entire ejection period, indicating that a mature perithecium does not become exhausted of spores as long as the canker remains wet.

Cankers moistened every 3 hours for 96 consecutive hours developed a spore ejection cycle which was possibly influenced both by the daily fluctuation in temperature and relative humidity and by the crowding of asci in perithecia. For one period of at least 60 hours duration, spore ejection was at a maximum every 12 hours.

The movement of ascospores away from a known source was subject to horizontal and vertical air currents within a stand, in addition to the mean wind direction. With wind speeds in excess of 10 m.p.h. spores remained close to the ground and with wind speeds of 5 m.p.h. or less updrafts carried spores vertically, while still only a short distance from the source. If hypoxylon ascospores moved in the same patterns as colored smoke, ascospores were apparently carried above the canopy a short distance, horizontally, from the source with winds less than 5 m.p.h. Spores carried by air movement above the stand might have a better chance for long distance dissemination than spores leaving the stand below the canopy at the leeward margin.

During a 4 year period, it was virtually impossible to completely eradicate hypoxylon cankers from an isolated aspen stand. Cankers were overlooked no matter how thorough the surveys, and rotorod samplers collected hypoxylon-like spores in the air originating either in the stand or from outside the stand.

Felling and sectioning aspen with hypoxylon cankers had the immediate effect of reducing new stromata production on cankers by 66 per cent in the first 10 months when compared with stromatal production on standing cankered aspen in the same period. Significant reduction of inoculum ejected from the felled cankers was not achieved until nearly 2 years after the trees had been felled.

Optimum temperature for the germination of ascospores, on a water agar medium buffered to pH 6.0, was 30-31°C with a range from

20 to 35°C. Optimum pH for germination was 5.7 with a range from 4.4 to 6.7. Three times as many ascospores germinated when the spores were placed on sapwood than on water agar, indicating that possibly open wounds are optimum infection locations. The phelloderm layer of aspen bark inhibited ascospore germination while the phellem and secondary phloem layers stimulated germination no more than did the water agar medium.

The bark moisture content of aspen is lower in the dormant season than during the growing season. During the dormant season there were no significant differences in average bark moisture contents between trees in stands with hypoxylon cankers and stands free of infection. Although the average relative turgidity percentages of the trees in all stands during October and November approached the moisture threshold level for hypoxylon growth in the bark (75-77 per cent), none of the average values dropped below 80 per cent. This indicated that during the dormant seasons of 1962-63 and 1963-64 no aspen stand in the study could be regarded as susceptible to hypoxylon attack solely on the basis of reduced bark moisture content. However, individual trees in these stands did, on occasion, have bark moisture contents below the threshold level. It is very possible that in other years the bark moisture content of a large number of aspen could drop below the threshold level and explain, in part, the increased number of infections which occur in some years and not in others.



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