

## Factors Important in Artificial Inoculation of *Pinus strobus* with *Cronartium ribicola*

Everett M. Hansen and Robert F. Patton

Former Research Assistant and Professor, respectively, Department of Plant Pathology, University of Wisconsin, Madison, WI 53706. Present address of senior author: Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331.

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison; by the National Science Foundation through appointment of the senior author as a NSF Fellow for 1971 and 1972; and by the McIntire-Stennis Project 1858. Appreciation is expressed to Steven A. Vicen for assistance in preparation of the illustrations.

Accepted for publication 18 March 1977.

### ABSTRACT

HANSEN, E. M., and R. F. PATTON. 1977. Factors important in artificial inoculation of *Pinus strobus* with *Cronartium ribicola*. *Phytopathology* 67:1108-1112.

Primary needles of eastern white pine were inoculated with basidiospores in an airstream apparatus and incubated in controlled-environment chambers to determine optimum conditions for infection. The amount and distribution of water on needle surfaces was critical to successful infection. Excess water caused spore clumping and abnormal germination. Exposure to light during incubation increased infection levels indirectly by improving moisture conditions

on the needles. Fluctuating temperatures and temperature shocks did not increase infection levels. Infection levels on inoculated seedlings, incubated in a dark dew chamber for 72 hr increased with spore density from 20 to 3,000 spores  $\text{mm}^{-2}$ . The highest spore densities resulted in 1.2 needle spots  $\text{mm}^{-2}$  of needle surface, with about 10% of the stomata successfully penetrated. Basidiospore germ tubes grew randomly on needles.

*Additional key words:* white pine blister rust, epifluorescence microscopy, infection efficiency.

White pine blister rust, which is caused by *Cronartium ribicola* J. C. Fischer ex Rabenh., is destructive to five-needle pines in North America, and control has been sought through genetic resistance. Several types of resistance mechanisms to this fungus have been identified in pine, a number of which are localized in the needles (1, 9). Determination of the nature of these needle reactions and factors that influence their expression requires reliable inoculation techniques that will yield abundant infections for comparative studies of susceptible and resistant selections.

Although the first artificial inoculations of white pines with *C. ribicola* were made in 1903 (7), there are no reports on inoculations with controlled levels of inoculum and, for years, tests for disease resistance have relied on mass inoculations. Hirt (6) first defined the approximate temperature and moisture requirements for infection, and Van Arsdell (12) made a critical determination of time, temperature, and humidity requirements. In the latter studies, success of infection in different trials varied from 18% to 100% without apparent explanation, and for years inoculation trials made by the junior author under various conditions have yielded erratic and inconsistent results. Limited knowledge of requirements for spore germination and penetration in the needle microenvironment prevent consistent attainment of multiple infections.

In previous work (4), basidiospore germination, germ tube development, and differentiation of infection structures were studied on artificial substrates and on

host needle surfaces. The present study applied this knowledge with the objective of obtaining maximum infection levels by artificial inoculation of eastern white pine (*Pinus strobus* L.) seedlings in controlled environment chambers. The effects of incubation conditions, germination behavior on the needle surface, and spore density were examined.

### MATERIALS AND METHODS

Eastern white pine seedlings were grown from open-pollinated seed in a growth room at 20 C, with a 12-hr photoperiod. Inoculations of primary needles were made on seedlings from 2-4 mo old.

The rust was maintained on *Ribes nigrum* L., and telia were produced on plants in a growth room at 16 C, as described previously (4).

Seedlings were inoculated in an airstream inoculation apparatus (Fig. 1) similar to one described by Snow (11) for work with *C. fusiforme*. Basidiospores, naturally released from telia on ribes leaves, were carried in an airstream and impacted in a discrete area on needles of a white pine seedling. Needles to be inoculated were held perpendicular to the airstream by a plastic template (Fig. 1-D and insert). Spore density on the needle surface was regulated by adjusting the period of exposure to the airstream.

Rate of spore deposition was a function of airflow rate and width of the receiving surface, as well as the number of basidiospores released from the ribes leaves. The spore flow rate was monitored by placing 1 x 5-mm strips of double-stick cellophane tape on toothpicks held in the plastic template in the airstream for 1-min exposures, and

then transferring the tape to a glass slide for a count of spores  $\text{mm}^{-2}$  (3). Length of inoculation exposures was adjusted to give a constant inoculum density according to

the spore flow rate. Spore deposition increased with airflow rate to  $23.5 \text{ liters min}^{-1}$ , but the deposition pattern was most uniform at  $19 \text{ liters min}^{-1}$ . Rates of spore

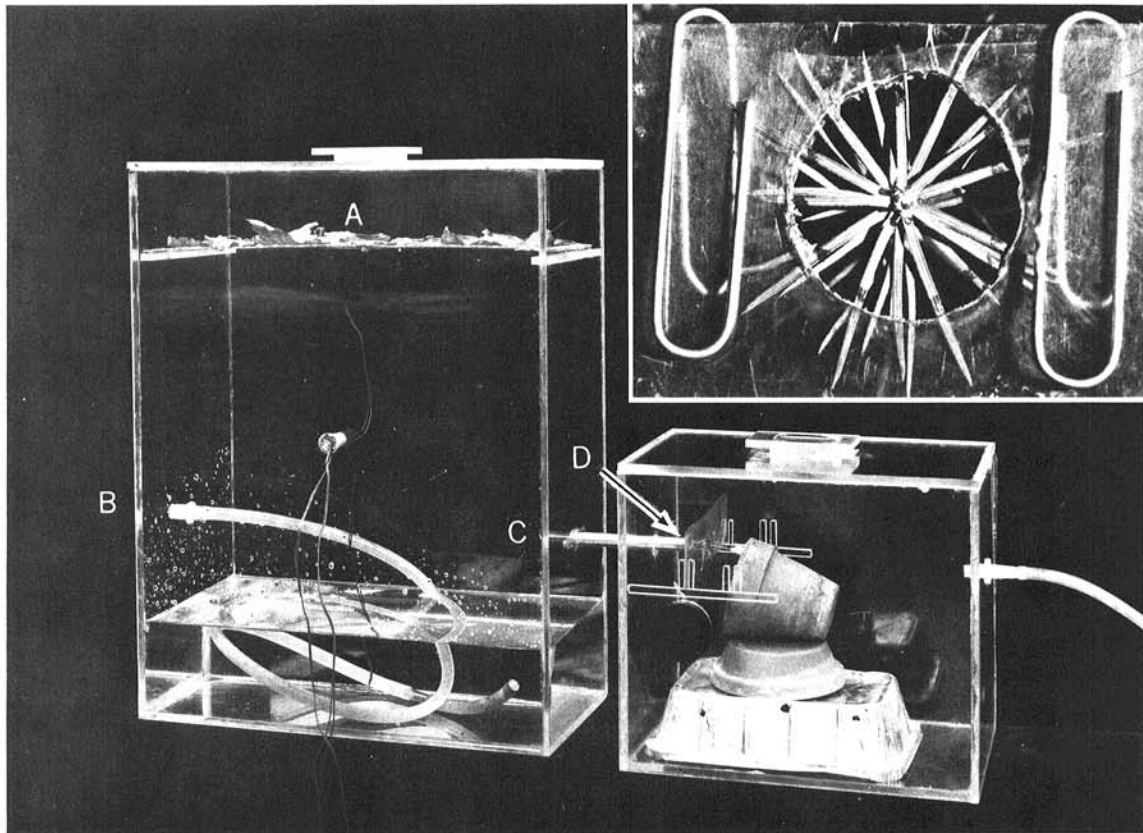


Fig. 2-(A to C). A) Needle spot on primary needle. Note distinct spot margins and basidiospore germ tube penetrating stoma. B) Needle spots on primary needles of one seedling 21 days after inoculation. C) Germinated basidiospores on needle surface. Note random orientation of germ tubes toward stomata.

deposition between 100 and 200 spores  $\text{mm}^{-2} \text{min}^{-1}$  were used for seedling inoculations.

Immediately after inoculation, seedlings were transferred to a dew chamber and held at 16 C under various moisture conditions for 72 hr, then transferred to a growth room at 20 C, for an additional 18 days or more for development of needle spot symptoms.

Observations of spore germination and counts of spore density were made by epifluorescence microscopy with a Leitz Ortholux microscope equipped with a Ploem Vertical Illuminator. Spores on the needle surface were stained with optical brighteners (4).

Counts, correlating substomatal vesicles of the rust with needle spots, were made on serial longitudinal 12- $\mu\text{m}$  sections of needles fixed in FAA and embedded in paraffin. Sections were mounted and observed unstained with phase contrast illumination.

## RESULTS

**Needle spot development.**—The first symptom of successful fungus penetration was a yellow spot centered on or adjacent to a stoma (Fig. 2-B). Needle spots were first visible between 17 and 20 days after inoculation. With epifluorescent illumination, needle spots appeared dull green in contrast to the red fluorescence of healthy tissue that surrounded the spots. The spore that initiated an individual infection could often be determined, and the course of its germ tube followed into the stomatal pit (Fig. 2-A).

The reliability of needle spots as a measure of the number of successful penetrations by basidiospore germ tubes was determined by microscopic examination of serial sections of 12 needles with a total of 50 needle spots. All needle spots had a pseudosclerotial mycelial mass in the underlying mesophyll tissue, and the infection hypha, marked by a substomatal vesicle, was found for 41 of the 50 spots. No infection hyphae were found that lacked vesicles, and only one vesicle was seen not associated with internal mycelium. In seven cases, separate penetrations of closely spaced stomata resulted in single spots.

**Effect of moisture and light during incubation.**—The amount and distribution of water on the needle surface during incubation was perhaps the most critical factor in basidiospore germination and needle infection. In dew chambers, dew droplets formed first on spore deposit areas, became visible within 4 hr, and continued to enlarge until they merged and fell from the needles. These conditions allowed maximum elongation of the germ tube, if the spore deposit remained undisturbed. Frequently, however, spores clumped around dew

droplets and sometimes were washed off the needle. Spores in clumps often did not germinate, or they germinated abnormally and formed secondary spores, thick germ tubes, or even zigzag germ tubes (4). This variation in needle surface moisture induced considerable variation in the amount of infection in a trial. For example, in one trial, each of nine seedlings was inoculated with 100 spores  $\text{mm}^{-2}$  and incubated in the dew chamber; the number of resulting needle spots on each tree ranged from 0 to 56.

Visible moisture on needles was not necessary for spore germination and infection. Visible dew did not form on inoculated trees that were covered immediately with a plastic bag and placed in the dew chamber. Variation in the amount of infection on covered trees was similar to that obtained from incubation with dew formation, but failures were caused by poor germination or short germ tubes, rather than clumped spores or abnormal types of germination.

Although several experiments were conducted to test the effect of light on infection, less dew formed with illumination, and it was not possible to separate light effects from moisture influences. Seedlings were incubated in two dew chambers with identical temperature and dew-formation settings. One chamber was illuminated through the window from a 200-W incandescent bulb behind a flowing water bath, 8 cm thick. Light intensity at seedling level was 3,766 lux. Wet- and dry-bulb temperatures, as measured by copper-constantan thermocouples at seedling level, were identical (16 C) for the light and dark chambers and did not change as the light was turned on and off. Dew formation, however, was consistently rated visually as moderate in the light chamber and heavy in the dark chamber. In the light chamber, spore deposits were more uniform, germination was more frequently normal rather than by thick germ tubes or secondary spores, and infection levels were higher than in the dark chamber. In the light, 1,197 needle spots developed on 28 seedlings, but in the dark, 561 spots formed on 29 seedlings.

**Effect of incubation temperature.**—Because changes in temperature greatly influenced the number and type of vesicles formed during germination of *C. ribicola* basidiospores on various substrates (4), fluctuating temperatures (10 to 21 C on a 12-hr cycle) and temperature shocks (2 hr at 21 C after a 16-hr period at 16 C, and then a return to 16 C) during incubation were tested for their effects on infection. During periods at 21 C, the chamber was lighted as described above.

Considerable variation in infection levels occurred within treatments. In most instances, low infection levels were correlated with clumping of spores on needles and resultant poor germination, apparently an influence of variable moisture levels on the needles. Eighty-nine trees were inoculated in three trials. In each trial, the number of needle spots per tree ranged from 0 to 40 or more. At a constant 16 C, the number of spots per tree averaged 13. Trees incubated with fluctuating temperatures averaged eight needle spots, and trees exposed to temperature shock averaged 13 needle spots. Although temperature shocks did not increase success of infection significantly, differentiation of vesicles on the needle surfaces was stimulated (3). Incubation at a constant 16 C in dark dew

TABLE 1. Effect of spore density on the infection of primary needles of *Pinus strobus* by *Cronartium ribicola*

Spores $\text{mm}^{-2}$	Spots $\text{mm}^{-2}$			Spores/spot
	Average	Range	Spots/100 stomata	
20 - 100	0.06	0.0 - 0.3	0.6	830
101 - 500	0.3	0.0 - 1.0	2.4	960
501 - 1,000	0.7	0.0 - 2.3	6.3	1,080
1,001 - 2,000	0.8	0.05 - 2.5	7.5	1,830
2,001 - 3,000	1.2	0.05 - 3.6	10.7	2,160



chambers was used routinely in subsequent experiments.

**Germ tube orientation.**—General observations of germ tubes on primary needles gave the impression that growth was random rather than perpendicular to the rows of stomata. The stomata of white pines occur in parallel rows, an arrangement superficially similar to the stomatal rows on wheat leaves. Directed growth of germ tubes seemed to be characteristic of several rust species (2), and Lewis and Day (8) reported that urediospore germ tubes of *Puccinia graminis* f. sp. *tritici* grew perpendicular to the stomatal rows, and thus increased the chances of encountering a stoma. Germ tubes from basidiospores of *C. ribicola* appeared to exhibit no such directed growth (Fig. 2-C). To verify this observation, a tally was made of whether the germ tubes were parallel or perpendicular to the stomatal rows. Crosshairs in one ocular of the epifluorescence microscope were centered on a spore being tallied, and the ocular turned to align the hairs at 45 degrees to the nearest stomatal row, thus dividing the observed needle surface into four quadrants. Germ tube growth was tallied as parallel or perpendicular to the stomatal rows, depending on the quadrant in which it occurred. In addition, germ tubes that changed general direction by more than 90 degrees were tallied as curved. A total of 430 germlings that were clearly visible from spore to germ tube tip on 15 needles of three trees were tallied. A  $\chi^2$  test showed no significant differences at  $P=0.10$  among the counts of 162 germlings parallel, 136 perpendicular, and 132 curved.

**Spore density and infection.**—Correlation of needle spot development with observations of spore germination on needle surfaces indicated that infection failure was most frequently because of poor spore germination and low spore densities. In a series of three tests with 123 seedlings, the relation between spore density and infection was determined. Exposure time in the airstream apparatus was varied to give spore densities on the needles of 20 to 3,000 spores  $\text{mm}^{-2}$ . After a 3-day incubation period, spore germination was checked by microscopic examination of sample needles, and only those seedlings with germinated spores (76 of 123) were held for needle spot counts. At least nine trees were in each of the five spore-density classes. Needle spots were counted on 10 needles (40  $\text{mm}^2$  of spore deposit area) per seedling (Table 1). These primary needles averaged 11 stomata  $\text{mm}^{-2}$  of upper needle surface, usually arranged in two parallel rows.

The variation in infection level in any spore-density class is perhaps the most important aspect of Table 1. On seedlings with fewer than the average number of infections, spores were often clumped and germination was abnormal. At spore densities above 500 spores  $\text{mm}^{-2}$ , all stomata in the inoculated areas had germ tubes over them and, at densities above 2,000 spores  $\text{mm}^{-2}$ , needles were covered with essentially a solid layer of spores.

The number of infections increased over the range of spore densities tested, but so did the average number of spores applied for each successful infection. At the lowest spore densities, seedlings averaged one needle spot for every 17  $\text{mm}^2$  of inoculated needle surface and, at the highest densities, one infection occurred for every 0.8  $\text{mm}^2$ . The ratio of spore density to needle spot density, an inverse measure of infection efficiency, increased from

one infection for every 830 spores at the lowest densities to one for every 2,160 spores at the highest density. The most heavily infected single seedling had 145 needle spots, an average of 3.6 spots  $\text{mm}^{-2}$  of inoculated needle area.

## DISCUSSION

The correlation between germination behavior of basidiospores on needles and subsequent infection levels supported previous conclusions about spore germination on collodion membranes (4). When most spores formed thick germ tubes or secondary spores, infection levels were low. Germination and infection processes were sensitive to small changes in temperature and moisture on the needles. Temperature was readily controlled in the dew chambers, but the amount of water on needle surfaces was not.

Successful penetration of a stoma nearly always resulted in a successful infection, but the growth of a germ tube through a stoma was apparently a chance event. The low infection success of basidiospore germ tubes may have been a direct result of their random growth. Urediospore germ tubes of *Puccinia graminis* f. sp. *tritici*, on the other hand, exhibited directed growth and differentiated an appressorium when they reached a stoma (8). As a consequence, one urediospore germ tube in four successfully penetrated wheat leaves (10), as compared to one in 720 for *C. ribicola* on our best single tree. On wheat at maximum infection levels, 44% of the stomata were penetrated, which compares favorably with 33% of stomata penetrated with maximum infection of white pine. In spite of the large amount of variation in infection level at any spore density, the average number of infections increased with spore density. However, at spore concentrations above 1,000  $\text{mm}^{-2}$ , infection efficiency rapidly declined. This may reflect a saturation of infection sites (stomata), or it may be an indirect indication that zigzag growth is reducing the infection potential of germ tubes at high densities (4).

The airstream inoculation system used in this work allowed reproducible quantitative inoculations, but was somewhat time consuming and cumbersome. However, repeated attempts to develop inoculation techniques with spray or needle injections, failed (3). Spore suspensions, atomized onto seedlings, resulted in erratic, low infection levels. Microscopic examination indicated that most spores failed to adhere to the needles, but those that did adhere usually formed secondary spores or thick germ tubes. Injection of concentrated spore suspensions directly into needles and cotyledons failed to produce needle spots. Needle tissue was infiltrated by the spore suspension, but no symptoms of infection developed, and microscopic examination of injected tissue revealed only ungerminated spores. Both spraying and injecting spore suspensions are successful techniques on slash pine with *Cronartium fusiforme* (5).

Although this study was based on inoculations of primary needles, more than 50 inoculations of secondary needles on older trees also were made. Germination behavior of spores was identical on the two needle types and needle spots developed nearly as rapidly and plentifully on elongating secondary needles as on primary needles. Our experience is, however, that needle spot

development on older secondary needles takes considerably longer, about 2-4 mo.

Further improvements in inoculating white pine with *C. ribicola* basidiospores will require a means to regulate more precisely the moisture levels on inoculated needles. The inoculation and incubation techniques described here should be sufficient to provide material for histological examination, if the needles are screened microscopically before sectioning. If only needles with uniform deposits of normally germinated spores are used, the chances of encountering successful penetrations will be improved.

#### LITERATURE CITED

1. BINGHAM, R. T., R. J. HOFF, and G. I. MC DONALD. 1971. Disease resistance in forest trees. *Annu. Rev. Phytopathol.* 9:433-452.
2. DICKINSON, S. 1969. Studies in the physiology of obligate parasitism. VI. Directed growth. *Phytopathol. Z.* 66:38-49.
3. HANSEN, E. M. 1972. Germination behavior and vesicle formation by *Cronartium ribicola* and the infection of *Pinus strobus*. Ph.D. Thesis, Univ. of Wisconsin, Madison. 118 p.
4. HANSEN, E. M., and R. F. PATTON. 1975. Types of germination and differentiation of vesicles by basidiospores of *Cronartium ribicola*. *Phytopathology* 65:1061-1071.
5. HARE, R. C. 1973. Correction: spray inoculation of slash pine with *Cronartium fusiforme*. *Plant Dis. Rep.* 57:1013-1015.
6. HIRT, H. H. 1942. The relation of certain meteorological factors to the infection of eastern white pine by the blister-rust fungus. *Bull. N.Y. State Coll. Forestry, Tech. Publ.* 59. 65 p.
7. KLEBAHN, H. 1905. Kulturversuche mit Rostpilzen. *Z. Pflanzenkr.* 15:65-108.
8. LEWIS, B. G., and J. R. DAY. 1972. Behavior of uredospore germ tubes of *Puccinia graminis tritici* in relation to the fine structure of wheat leaf surfaces. *Trans. Br. Mycol. Soc.* 58:139-145.
9. PATTON, R. F. 1967. Factors in white pine blister rust resistance. Pages 876-890 in *Proc. XIV IUFRO-Kongress, Section 22/24. München, Germany.*
10. PETERSEN, L. J. 1959. Relations between inoculum density and infection of wheat by uredospores of *Puccinia graminis var. tritici*. *Phytopathology* 49:607-614.
11. SNOW, G. A. 1968. Basidiospore production by *Cronartium fusiforme* as affected by suboptimal temperatures and preconditioning of teliospores. *Phytopathology* 58:1541-1546.
12. VAN ARSDEL, E. P. 1968. Stem and needle inoculations of eastern white pine with the blister rust fungus. *Phytopathology* 58:512-514.