Mode of Penetration of Needles of Eastern White Pine by Cronartium ribicola

Robert F. Patton and David W. Johnson

Professor, and former Project Assistant, now Graduate Research Assistant, respectively, Department of Plant Pathology, University of Wisconsin, Madison 53706.

Published with approval of the Director, Wisconsin Agricultural Experiment Station. Supported in part by U.S. Forest Service Grant No. 1 (4000) and National Science Foundation Grant GB-3297.

Accepted for publication 23 January 1970.

ABSTRACT

Labeling basidiospores of *Cronartium ribicola* with a fluorescent brightener facilitated detection of penetration sites of eastern white pine needles by the germ tubes. Entry of the fungus was through the stomata. The germ tube of a basidiospore on the needle surface grew into the outer stomatal pit, penetrated between the guard cells as a very thin strand, and then characteristically formed a substomatal vesicle and an infection hypha from which a mycelium developed in the mesophyll. Germ tubes that grew over the surface of the needle were brightly fluorescent for their entire length. All fluorescence imparted by the fluorescent brightener was quenched at the point of entry between the guard cells, indicating some host-parasite interaction at this point. Phytopathology 60:977-982.

Research at the University of Wisconsin on resistance to white pine blister rust has been carried on since 1937. First efforts were devoted to determining whether resistance to the inciting fungus, *Cronartium ribicola* J. C. Fisher ex Rabenh., existed in the native eastern white pine, *Pinus strobus* L., and later to selection and breeding for resistance (8, 12, 13, 15, 16). This work established a foundation on which to base studies on the nature of resistance, pathogen variability, and influence of environment and host variation on establishment of infection and expression of host resistance. In recent years our work has been directed toward a clarification of the process of needle infection in susceptible and resistant hosts. The purpose of this paper is to report our observations on the mode of penetration of needles of eastern white pine by the rust fungus, *C. ribicola.* A preliminary report has been published (10).

Little is known of the histology of pine needle infection by *C. ribicola,* and some reports are contradictory. The experiments of Clinton & McCormick reported in 1919 (2) were the first to give indications of the mode of entry into white pine needles. They described and illustrated substomatal vesicles which they felt were the result of stomatal penetration by the fungus, but they were never able to verify this belief by observing a hyphal connection between spores on the needle surface and the vesicle within the needle. Nor did they see any evidence of penetration directly through the cuticle and into or between the epidermal cells. In contrast to these observations, Hirt (5) reported several instances of direct penetration of epidermal cells. Unfortunately his account was brief and without illustration. He did see substomatal vesicles in sections of established needle infections, but indicated that their nature and significance still remained to be determined. In a mimeographed Interim Report, Boyer (1) stated that penetration by the sporidia into the primary leaf or hypocotyl appeared to be directly through the cuticle by a fine penetration hypha. Again he could not observe any direct connection between a spore on the needle surface and the hyphae in infected mesophyll cells. Frequent penetrations "at 5 days" on hypocotyls were reported, although infections never developed. Even Patton & Nicholls (11), in a report presented in 1964, made a tentative interpretation of possible direct penetration of the cuticle and epidermis in two out of many thousands of observed sections of inoculated needles.

MATERIALS AND METHODS.—*Cronartium ribicola* was maintained on plants of *Ribes nigrum* L. in a greenhouse or sometimes collected on leaves from cultivated plants of this currant remaining in gardens in southern Wisconsin.

Needles for microscopic examination were obtained from trees inoculated artificially either in nursery beds outdoors, in moist chambers in a greenhouse, or in a controlled-environment growth chamber. Eastern white pines used for inoculation were seedlings from 1 to about 6 months old grown in the greenhouse from seed, ordinary nursery-run 4-year-old transplants, and 4- to 6-year-old grafts from known susceptible and resistant selections ranging from approximately 30-50 years old. For inoculation, telia-bearing ribes leaves were attached, for a 72-hr period, to the leader or branches so that basidiospores would be cast onto the current season's needles. Nursery beds were enclosed with muslin- or burlap-covered frames, and the chambers were kept wet constantly with fine water sprays. Such inoculations were made in late summer or fall when temp within the frames usually fluctuated somewhere within a range of approximately 4-28 C. In the greenhouse, inoculations were conducted in a mist chamber where temp was maintained at 20 ± about 2.5 C. In the growth chamber, trees were held under a wet-burlap-covered frame over a pan of water; relative humidity within the burlap chamber remained at 100%, and temp of the growth chamber was regulated to give a diurnal fluctuation between approximately 4.5-24 C. The minimum inoculation period was 72 hr, but occasionally trees remained in the chambers for as long as 120 hr. Needle collections were made at the end of the inoculation period and at various intervals thereafter up to 10 months.

In order to study needle penetration and subsequent infection, it was essential to locate penetration sites easily. The usual histological staining procedures did not always give the good contrast necessary for the intensive screening essential to the location of initial

infection sites. Such necessary contrast and precise location of the fungus on the needle was obtained by labeling basidiospores with a fluorescent brightener and examining needle sections with a fluorescence microscope. This technique was described in a previous article (11). As the brightening agent, we used a derivative of di-amino stilbene disulfonic acid [the disodium salt of 4,4′-bis-4-anilino-6-bis (2-hydroxyethyl) amino-*s*-triazin-2-ylamino-2-2′-stilbenedisulfonic acid] received as a 12% solution in 42% aqueous cellosolve from the American Cyanamid Company. This is one of many such compounds known as optical bleaches or brighteners, and is used in industry for brightening paper and textiles. A concentration of 0.03 ml of stock solution in 50 ml of distilled or deionized water was chosen as a standard. The labeling technique consisted of wetting teliospores with the brightener solution either by spraying the leaf or soaking it for a few min. Excess brightener solution was shaken off, or the leaf was lightly blotted with paper toweling to remove excess moisture from the teliospores and to avoid having droplets of the brightener contact the needles. In later experiments, spores on the needle surface sometimes were brightened or rebrightened by soaking needles bearing a spore cast in a solution of the brightener; the needle cuticle did not absorb the brightener, and good contrast between the fungus and needle tissue was attained. Upon germination of the teliospores, the brightener passed on to the basidiospore and persisted in a concentration high enough to brighten even rather long germ tubes. In a beam of near-ultraviolet light from the microscope lamp, deposits of brightened basidiospores cast onto pine needles were detected as patches of bluish fluorescence, and these segments of the needle were then cut out for fixing and sectioning by the paraffin method (6).

Standard histological procedures were followed for sectioning and mounting. No staining was necessary, and low-fluorescence mounting media were used. Once the paraffin was removed, sections were hydrated to 35% alcohol and mounted in buffered glycerine or an Elvanol semipermanent mounting medium (17).

Observations were made with a Zeiss microscope equipped with Neofluar objectives and a bright field condenser. Illumination for fluorescence microscopy was from an Osram high-pressure HBO 200-w lamp, used with a UG I exciter filter (Schott) and barrier filters —65 and 41. For photomicrographs, Ansco 200 35-mm film was used for color slides, while Adox KB14 or Polaroid films were used for black and white.

RESULTS.—*Fluorescence of labeled spores.*—The basidiospores that carried the brightener fluoresced with a brilliant blue color that contrasted well with the blue-green and green to amber or orange autofluorescence of the needle sections. Peak fluorescence of the brightener was at a wavelength of 428 mμ as measured by a Fluorespec Model SF-1 fluorescence spectrophotometer. The fluorescence of the stained spores or their germ tubes attracted the eye immediately, and greatly facilitated examinations of the needle sections.

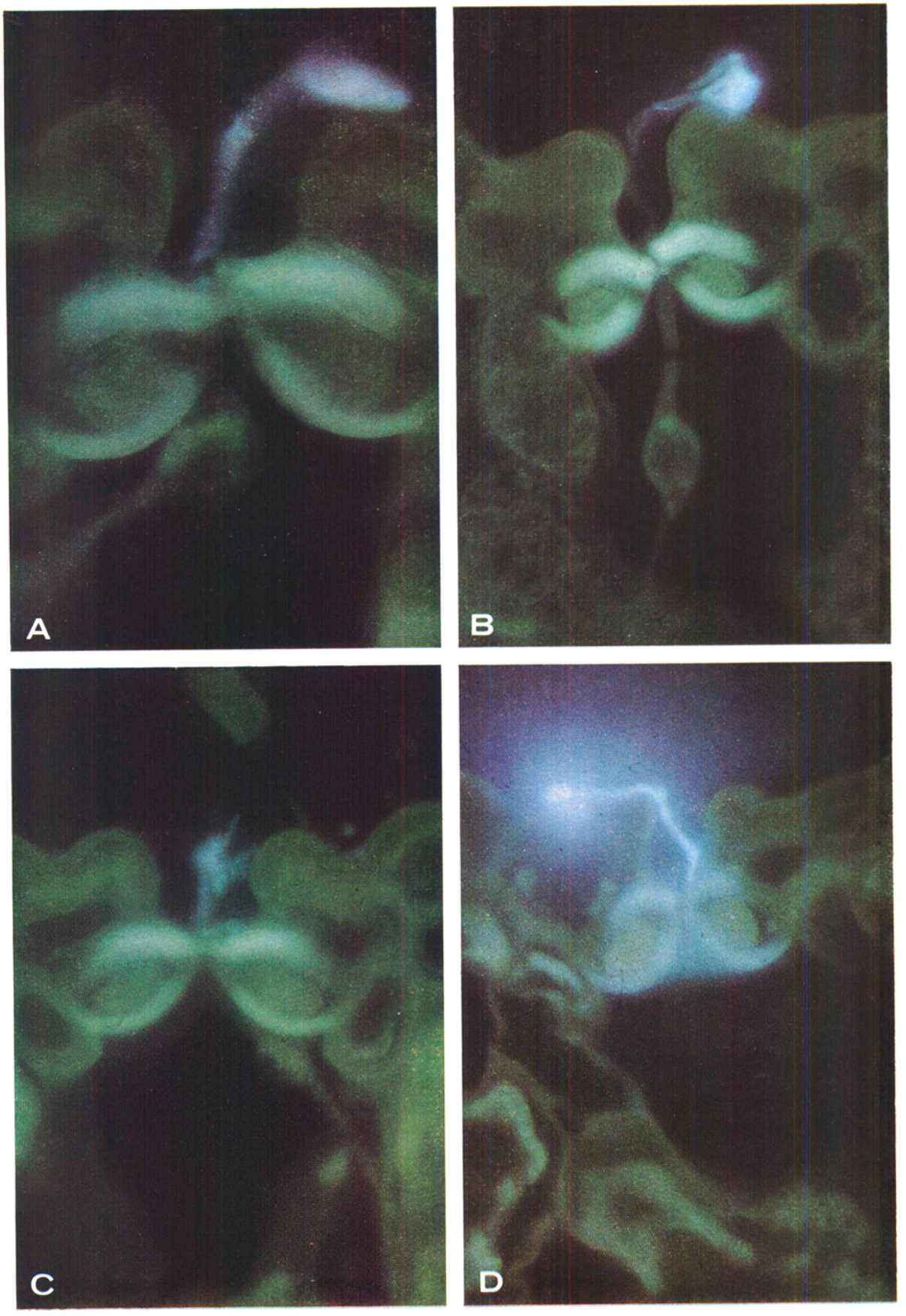
Mode of penetration.—Entry of the fungus into the needle through the stomata, as believed to occur by Clinton & McCormick (2), was confirmed by observations of hundreds of sites of initial infection. This manner of entry was consistently the same from both indoor and outdoor inoculations, and for all types and ages of needles from susceptible pines, including cotyledons, primary needles, and secondary needles of both young and old trees. No direct penetration of cuticle and epidermis was observed.

The germ tube of a basidiospore on the needle surface grew into the stomatal pit, penetrated between the guard cells as a very thin strand, and subsequently developed into a substomatal vesicle in the chamber beneath the guard cells. Usually the vesicle formed very close beneath the guard cells (Fig. 1-A), but sometimes the germ tube continued some growth before formation of the vesicle well down into the substomatal chamber (Fig. 1-B). The vesicles varied in shape from almost globose to fusiform-elliptical, ovate or dacryoid shapes being the most common (18). The description and illustrations of vesicles by Clinton & McCormick (2) are representative of the typical vesicles seen in the present work. Sometimes the vesicle narrowed to a sharp, beaklike extension (Fig. 2) when it developed immediately after passage of the narrowed germ tube between the guard cells. Other vesicles appeared to have developed as a swelling of a germ tube that attained normal size again in the substomatal chamber (Fig. 1-A, B). In the early stages of infection, the wall of the vesicle was similar to that of the germ tube or infection hyphae, but occasionally, vesicles in 7- or 10-month-old infections appeared thick-walled (Fig. 3, 4). The persistence of the vesicle permitted identification of the initial penetration point by a germ tube from a single spore. Two vesicles in a single chamber, indicating infection by two spores, were seen in a few sections of artificially inoculated needles (Fig. 5).

From the lower portion of the vesicle, a single hypha grew across the chamber to the mesophyll cells (Fig. 1-A, B, C). This hypha, called the primary hypha by Clinton & McCormick (2), is equivalent to the in-

→

Fig. 1. Fluorescence photomicrographs of penetration of stomata of eastern white pine needles by *Cronartium ribicola*. The blue fluorescence of the spore and germ tube was imparted by the fluorescent brightener; fluorescence of the guard cells, other cells of the needle, and the substomatal vesicle and infection hypha of the fungus is natural. **A)** Stomatal penetration of a secondary needle collected 5 days after inoculation began, with formation of substomatal vesicle close to guard cells. **B)** Stomatal penetration of a primary needle collected 38 days after inoculation began, with formation of vesicle well within substomatal chamber. **C)** Stomatal penetration of a secondary needle collected 5 days after inoculation began. Germ tube grew horizontally over surface of guard cell before it penetrated. There is no appressorium. Note lack of fluorescence by brightener in germ tubes, vesicles, and infection hyphae in A, B, and C once the germ tube has passed between the guard cells, indicating a degradation of the brightener at this point. **D)** Germ tube grew to the surface of the guard cells but did not penetrate further into the needle.


A
B
C
D

fecting hypha of Pole-Evans (14) and the infection hypha mentioned by several subsequent workers concerned with cereal rust spores (e.g., 3, 4). This hypha was almost always nonseptate. Septations became common only in the branching mycelium that developed from this infection hypha in the mesophyll tissue.

No appressorium was seen in the outer stomatal pit before the germ tube entered between the guard cells. Occasionally the germ tube grew horizontally across the bottom of the pit before penetrating the stoma. A longitudinal view of this portion of a germ tube might easily be misinterpreted as an appressorium (Fig. 1-C).

In a number of stomata, germ tubes were observed in the stomatal pit but there had been no penetration between the guard cells into the substomatal chamber (Fig. 1-D). At these sites, for some unknown reason, germ tube development ceased at the top of the guard cells.

Collections of inoculated needles made at various intervals after inoculation contained vesicles and infection hyphae. The earliest collections were made at 5 days after the beginning of the inoculation, and Fig. 1-A and C are typical of the stage of development of the fungus at this time. With such early collections, there was a greater likelihood of finding vesicles still connected to the germ tube and spore, but Figure 1-B shows a spore, germ tube, vesicle, and infection hypha in a primary needle 38 days after the beginning of the 3-day inoculation period. After inoculation outdoors, the seedlings in this group had been held in the greenhouse. The mycelium that developed from the infection hypha had by this time begun to ramify through the mesophyll. A vesicle, once formed, was persistent and could usually be located easily in 7- and 10-month-old needle infections (Fig. 3), which by that time were readily evident as needle spots.

Subsequent mycelial development.—Once the infection hypha branched, it continued to develop into a much-branched, septate mycelium. Haustoria were formed in mesophyll cells. As the tissue broke down, vegetative hyphae also invaded the cells. In primary needles the mycelium soon developed into compact masses (Fig. 6), in one collection as early as 9 days after inoculation. In secondary needles, by 21 days the infection hypha had branched to form two or three main hyphae (Fig. 7), but by 49 days compact, sclerotialike masses, as described by Clinton & McCormick (2), had been formed in the mesophyll. In cross sections these masses appear nearly circular, whereas in longitudinal sections they are several times longer than they are wide (Fig. 8). As hyphal development continues, pressure of the mycelial mass often flattens one or more cells of the endodermis (Fig. 8, 9). The endodermal ring is thus broken, and the mycelium then proliferates into the stelar tissues (Fig. 9).

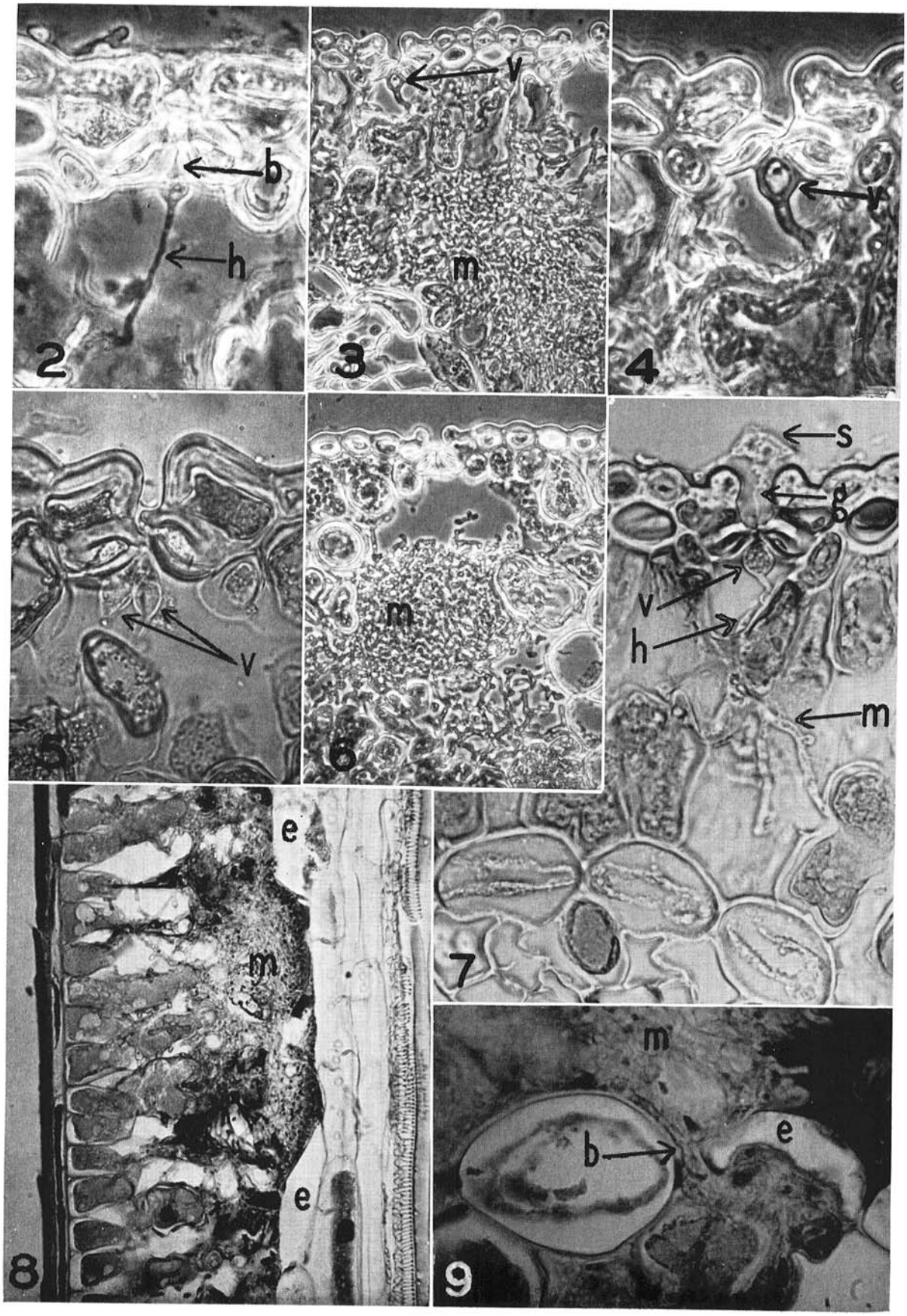
Invasion of the stele was not studied in detail, but observations incidental to the study of stomatal penetrations indicated that the fungus usually grew for many weeks or months before it broke through the endodermis. In collections taken 7 months after inoculation, dense masses of mycelium had formed in the mesophyll. In one needle, the mycelium from three separate infections, traced from the substomatal vesicles, had merged into one mass approximately 950 μ long. At this stage the fungus was not seen in the vascular core. In several sections, the mycelial mass had depressed some of the endodermal cells and deformed the ring of endodermis, but had not penetrated into the endodermis and had not broken through into the vascular core. In some other needles from this 7-month collection, pressure from the mycelial mass had caused separation between cells of the endodermis, and the mycelium had invaded the stele. Various stages of stelar invasion were seen, from initial penetration of a few hyphae into the transfusion tissue to development of dense masses of mycelium in localized areas of the transfusion tissue of the stele. Occasionally, even cells of the endodermis were invaded. Further development into the phloem and thence on down to the stem, as reported by Clinton & McCormick (2), was not followed in this work.

Quenching of fluorescence.—Germ tubes that grew over the surface of the needle or into the stomatal pit were brightly fluorescent for their entire length (Fig. 1-D). At the point where the germ tube passed between the guard cells, however, the fluorescence imparted by the fluorescent label was quenched, and none of the subsequently formed infection structures, including continuation of the germ tube, the vesicle, and the infection hypha, showed any of the blue fluorescence of the brightener (Fig. 1-A, B, C). These structures usually could be seen by their faint natural fluorescence ranging from shades of dull green or brown to a dull blue-gray. The contrast between the brilliant blue of the spore and germ tube on the outside of the needle and the dull natural fluorescence of the fungus once it was within the needle was quite striking.

Influence of resistance.—When penetration of needles of one resistant selection occurred, the manner of entry and subsequent development of the fungus was the same as in needles of susceptible trees. In needles of another highly resistant selection, No. 327, no typical penetrations were observed. Here the germ tube grew down into the outer stomatal pit but failed to continue growth between the guard cells and develop the typical substomatal vesicle and infection hypha.

DISCUSSION.—From this work it is clear that entry of *C. ribicola* into the white pine needle is through a stoma, with the subsequent characteristic production of a substomatal vesicle and infection hypha. All the previous reports of direct penetration (1, 5, 11) are now believed to be misinterpretation of artifacts produced in sectioning the needles, such as minute cracks, strain lines, or other optical effects in the cuticle or cell wall.

Clarification of the mode of penetration of the white pine needle should help define in future work the sites of host-parasite interactions that should be investigated in examining the mechanisms involved in needle resistance. Quenching of fluorescence of the brightened germ tube at the point where it passed between the guard cells of the stoma was indicative of some interaction between host and parasite at this point. The hypothesis has been proposed (9) that one expression of


b
h
2
v
m
3
v
4
v
5
m
6
s
g
v
h
m
7
e
m
e
8
m
b
e
9

←

Fig. 2-9. Development of *Cronartium ribicola* in needles of eastern white pine after stomatal penetration. **2)** Phase contrast photograph of beak (b) of vesicle formed immediately beneath guard cells. Infection hypha (h) has penetrated mesophyll cell (×680). **3)** Thick-walled vesicle (v) and mycelial development (m) from it in the mesophyll in a 7-month-old infection of a secondary needle—phase contrast (×270). **4)** Close-up of the vesicle (v) in Fig. 3 (×680). **5)** Two vesicles (v) in the same substomatal chamber of a primary needle collected 15 days after inoculation began—unstained, bright-field illumination (×680). **6)** Mycelial mass (m) in mesophyll of a primary needle collected 38 days after inoculation began—phase contrast (×270). **7)** Stage of development in a secondary needle collected 21 days after inoculation began—unstained, bright field. A germ tube (g) from one of two basidiospores (s) deposited on the edge of the stoma formed a substomatal vesicle (v) immediately beneath the guard cells and an infection hypha (h) that branched to form two main strands (m) as the beginning of a mycelium in the mesophyll (×500). **8)** Longitudinal section of a secondary needle collected 7 months after inoculation—stained, bright field. Mycelial mass (m) that developed in the mesophyll collapsed a portion of the endodermis (e) but at this point had not penetrated into the vascular core (×270). **9)** Cross section of a secondary needle collected 7 months after inoculation—stained, bright field. Mycelium (m) had massed in the mesophyll, crushed an endodermal cell (e), and broken through into the vascular core at point (b) between two separated cells of the endodermis (×680).

needle resistance may be the inhibition of vesicle formation and subsequent host infection by some host-parasite interaction in the stomatal area.

The influence of resistance on initial penetration of the needles seems to be variable. Whether or not a germ tube penetrates between the guard cells and forms a substomatal vesicle and infection hypha may be determined partly by whether a tree is inherently resistant and partly by the nature of the resistance. Concomitant with this study of mode of penetration, comparisons were made of the number of vesicles observed in sections of measured lengths of inoculated needles of susceptible and resistant selections (9). Although in needles of one resistant selection (No. 30) a few vesicles were observed, in another highly resistant selection (No. 327) no vesicles were seen in examinations of sections from over 24 cm of inoculated needle length. This difference is probably explained by the existence of at least two different types of resistance: needle resistance and bark resistance. In past work, our rating of selections as resistant has been on the over-all basis of response to artificial inoculation in terms of final infection and bark lesions. Thus, some selections were rated as "resistant" that had needle spots (the result of typical infection of needles) but no cankers or small incipient cankers that were soon corked-out as a result of bark resistance. In this study of penetration, selection No. 30 is one that may have much less resistance to needle infection than the highly resistant selection No. 327, but does have resistance to establishment of the fungus in bark tissues. Reduction in number of vesicles, possibly even to zero, is one aspect of genetic resistance to initial infection of needles.

In some recent unpublished work we have obtained evidence that wax plugs in stomata can influence the amount of infection. Wax plugs often prevented growth of germ tubes down into the outer stomatal pits, and consequently reduced the number of opportunities for entry into the substomatal chamber and infection of needles even of inherently susceptible trees. We know that susceptibility of eastern white pine to infection decreased with age (7), and that the number of penetrations of the needle by the rust was markedly less with increasing age of the tree (9, 13). Since occlusion of stomata by wax is apparently related to type of needle and age of tree, this stomatal effect may be one explanation for the influence of age on infection, although it is not considered a major mechanism of resistance.

LITERATURE CITED

1. Boyer, M. G. 1962. Studies on white pine blister rust. Interim Report, Can. Dep. Forest., Forest. Entomol. and Pathol. Branch, Forest. Pathol. Lab., Maple, Ontario. 38 p.
2. Clinton, G. P., & F. A. McCormick. 1919. Infection experiments of *Pinus strobus* with *Cronartium ribicola*, p. 428-459. Report of the Botanist for Years 1917-18, Conn. Agr. Exp. Sta. Bull. 214.
3. Dickinson, S. 1949. Studies in the physiology of obligate parasitism. II. The behaviour of the germ tubes of certain rusts in contact with various membranes. Ann. Bot. (N.S.) 13:219-236.
4. Emge, R. G. 1958. The influence of light and temperature on the formation of infection-type structures of *Puccinia graminis* var. *tritici* on artificial substrates. Phytopathology 48:649-652.
5. Hirt, R. R. 1938. Relation of stomata to infection of *Pinus strobus* by *Cronartium ribicola*. Phytopathology 28:180-190.
6. Johansen, D. A. 1940. Plant microtechnique. McGraw-Hill Book Co., N. Y. 523 p.
7. Patton, R. F. 1961. The effect of age upon susceptibility of eastern white pine to infection by *Cronartium ribicola*. Phytopathology 51:429-434.
8. Patton, R. F. 1966. Interspecific hybridization in breeding for white pine blister rust resistance, p. 367-376. *In* H. D. Gerhold et al. [ed.] Breeding pest-resistant trees. Pergamon Press, N. Y.
9. Patton, R. F. 1967. Factors in white pine blister rust resistance, p. 876-890. XIVth IUFRO Congress Proc., Sept. 4-9, 1967, Munich, Germany. Part III, Section 22, and Intersectional Working Group 22/24.
10. Patton, R. F., & D. W. Johnson. 1966. Penetration of pine needles by *Cronartium* basidiospores labeled with a fluorescent brightener. Phytopathology 56: 894 (Abstr.).
11. Patton, R. F., & T. H. Nicholls. 1966. Fluorescent labeling for observation of basidiospores of *Cronartium ribicola* on white pine needles, p. 153-162. *In* H. D. Gerhold et al. [ed.] Breeding pest-resistant trees. Pergamon Press, N. Y.
12. Patton, R. F., & A. J. Riker. 1958. Blister rust resistance in eastern white pine, p. 46-51. 5th Northeastern Forest Tree Improvement Conf. Proc., 1957.
13. Patton, R. F., & A. J. Riker. 1966. Lessons from nursery and field testing of eastern white pine selections and progenies for resistance to blister rust, p. 403-414. *In* H. D. Gerhold et al. [ed.] Breeding pest-resistant trees. Pergamon Press, N. Y.
14. Pole-Evans, I. B. 1907. The cereal rusts. I. The development of their Uredo mycelia. Ann. Bot. 21:441-466.

15. RIKER, A. J., T. F. KOUBA, W. H. BRENER, & L. E. BYAM. 1943. White pine selections tested for resistance to blister rust. J. Forestry 41:753-760.
16. RIKER, A. J., & R. F. PAITON. 1961. Breeding trees for disease resistance, p. 1687-1691. Recent Advances in Bot., Sect. 14. Univ. Toronto Press, Toronto.
17. RODRIGUEZ, J., & F. DEINHARDT. 1960. Preparation of a semi-permanent mounting medium for fluorescent antibody studies. Virology 12:316-317.
18. SNELL, W. H., & E. A. DICK. 1957. A glossary of mycology. Harvard Univ. Press, Cambridge, Mass. 171 p.