Histopathology of Colonization in Leaf Tissue of *Castilleja*, *Pedicularis*, *Phaseolus*, and *Ribes* Species by *Cronartium ribicola*

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ABSTRACT

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The white pine blister rust fungus has been recognized as comprising two formae speciales, *Cronartium ribicola* f. sp. *ribicola* and *C. ribicola* f. sp. *pedicularis*, each with different alternate hosts and different expressions of pathogenicity to some pine species. We observed interactions of a Wisconsin isolate of *C. r. ribicola* with a conventional alternate host, an alternate host commonly infected by the East Asian form (*C. r. pedicularis*), and plants considered as nonhosts for our North American form. Leaf samples collected at different times after inoculation with urediniospores were examined by light microscopy for penetration and infection or colonization. In the conventional alternate host, *Ribes nigrum*, the fungus achieved an established infection through the formation of haustoria, which were first visible 6 days after inoculation. The development of typical symptoms and signs followed within 7–9 days after inoculation. In *Pedicularis resupinata*, the host for the East Asian form, penetration and early stages of colonization were similar to those stages in *R. nigrum*. Infection hyphae branched sparingly in the intercellular spaces and occasionally reached as far as the upper epidermis between 5 and 16 days after inoculation but did not develop further. Colonization in the native *P. canadensis* and the common bean, *Phaseolus vulgaris*, was similar to but slightly less extensive than in *P. resupinata*. Colonization in localized portions of leaves of some plants of *Castilleja miniata* was much more extensive than in the other nonhost species. In leaf sections taken 63 days after inoculation, intercellular space, in portions up to 2 mm long, was densely packed with profusely branched hyphae. Haustorial mother cells developed in all species, but no haustoria were formed in any species except *R. nigrum*. The results supported previous indications of a difference in pathogenicity between the two forms of *C. ribicola*. The surprising amount of colonization in *C. miniata* led to speculation about the possible presence of the East Asian form of the rust in the Pacific Northwest, or the possibility of adaptation of *C. miniata* as an alternate host for our North American form.

In tests of resistance to white pine blister rust in Europe and North America, caused by *Cronartium ribicola* J. C. Fischer ex Rabenhorst, *Pinus koraiensis* S. & Z. has always been rated as highly resistant (2,16). In eastern Asia, however, *P. koraiensis* has suffered considerable damage from outbreaks of a blister rust similar to the typical white pine blister rust. Development of the disease in Korea particularly was reported by La and Yi (18), and through artificial inoculations they proved that species of *Pedicularis* and *Ribes* served as alternate hosts of this rust. Under natural conditions the pathogen alternates between *P. koraiensis* and *Pedicularis resupinata* L. Formerly, a rust fungus on *Pinus pumila* Regel in Japan was recognized as *C. kamtschaticum* Jørstad, and species of *Pedicularis* and *Castilleja* were thought to be the telial hosts, although apparently this connection had not been proved by artificial inoculations (30). Yokota and Uozumi (31), on the basis of artificial inoculations with *Cronartium* stem rusts of *P. pumila* and *P. strobus* L. in Japan, considered that *C. ribicola* is a collective species, and included *C. kamtschaticum* as a synonym. They tentatively proposed a classification of *C. ribicola* into two formae speciales, with *C. r. pedicularis* having as alternate hosts *Pedicularis* spp. and *Ribes* spp., and *C. r. ribicola* alternating to *Ribes* spp. The relatively common occurrence of *C. ribicola* on *P. koraiensis* in eastern Asia strongly suggests that the form species in eastern Asia has different pathogenicity than the rust commonly encountered in the United States. In 1983 Yokota (30) reported on tests in Japan of resistant progenies of *P. monticola* Dougl. developed in the U. S. Forest Service resistance program in Idaho. When inoculated with a rust that had *P. resupinata* as its main telial host, these progenies showed no resistance to the race of rust present in the Hokkaido area. Also, Stephan and Hyun (28) reported on a cooperative experiment in which *R. nigrum* L. and *P. resupinata* both were inoculated with European and East Asian sources of *C. ribicola*. In West Germany, aeciosopores from naturally infected *P. strobus* in northern Germany infected only *R. nigrum*; in South Korea aeciospores from naturally infected *P. koraiensis* infected only *P. resupinata*. The authors suggested that there are strains of *C. ribicola* with different pathogenicities. They discussed the possible existence of four separate types of the rust and problems of identification and taxonomic status of these special forms. Though they believed these four types could be described as four formae speciales, they refrained from assigning these forms taxonomic ranks.

Ribes spp. usually have been considered as the only alternate hosts for the form of *C. ribicola* present in North America. These new views of the different pathogenic capabilities of forms of *C. ribicola* open consideration that other species act as alternate hosts for another form of rust on this continent. Evidence along this line was the report in 1976 by Hiratsuka and Maruyama (15) on infection and development of telia on *Castilleja miniata* Dougl. ex Hook. after inoculations in the greenhouse with aeciospores of *C. ribicola* from *P. monticola* from British Columbia and from *P. albicaulis* Engelm. from Alberta, Canada. Subsequently, Hunt (17) evaluated the importance of Scrophulariaceae as alternate hosts for *C. ribicola* under field conditions. After inoculations at five different locations in the field of three species of *Pedicularis*, two of *Castilleja*, and of *Rhinanthus crista-galli* L., and a greenhouse inoculation of *C. miniata* at Victoria, BC, no symptoms or signs were evident on any of the plants.

In a preliminary report of a study of host-parasite interactions with a form of *C. ribicola*, Patton and Spear (22) described colonization of *R. nigrum*, *P. canadensis*, *P. resupinata*, and *Phaseolus vulgaris* L. 'Eagle' after inoculations with urediniospores of *C. ribicola* from Wisconsin. Within 18 hr after inoculation,

when a germ tube encountered a stoma on a leaf of any of these species, often an appressorium developed over the stoma. Within 24 hr after inoculation an infection hypha penetrated between the guard cells into the substomatal chamber. In *R. nigrum*, by 5 days after inoculation colonization of intercellular spaces above the entry point had occurred by branching growth of hyphae along with some localized lateral spread. Often, by 7 days uredinia could be discerned as tiny yellowish blisters forming beneath the lower epidermis and these were sporulating by 9 days. After 16 days the fungus had colonized much of the intercellular space around infection centers, and haustoria were readily observed in mesophyll cells. In *P. resupinata,* early stages of the infection process were similar to those in *R. nigrum*, but by 5 days after inoculation the infection hypha usually had only grown into the intercellular space between cells of the spongy mesophyll, and there was less branching and less extensive colonization than in *Ribes*. In a few sections the infection hypha had reached the upper epidermis but branching was not extensive. The tip of the infection hypha, and often of one to several hyphal branches, was slightly swollen and delimited by a septum. Development of the fungus in *P. canadensis* and *P. vulgaris* was essentially the same as that in *P. resupinata*. In none of the hosts, except *R. nigrum*, were any signs or symptoms evident to the unaided eye, nor were any haustoria seen.

To contribute further to our understanding of the pathogenic capabilities of forms of the rust, the present paper summarizes additional observations of the histopathology of the Wisconsin strain of *C. ribicola* on the species previously examined, and also describes the extensive colonization in leaves of *C. miniata*.

MATERIALS AND METHODS

Host plants. Rust development on *R. nigrum*, the European black currant, a very susceptible species, served as the standard for comparison of rust behavior on the other species. These plants were rooted cuttings from stocks that have been used for many years at the University of Wisconsin-Madison.

Seeds of *P. resupinata* L., an Asian member of the Scrophulariaceae, were obtained from People's Republic of China, Japan, and South Korea. The plants are root parasites and have been difficult to grow in our growth rooms. Thus, we were unable to inoculate leaves that were always of a certain age. Our final system of growing the plants included germination of surface-sterilized seed on moist filter paper or 3% water agar. Then, after development of cotyledonary leaflets, seedlings were transplanted to pots of sterile medium such as a commercial potting mixture or a mix of loam-sand (50:50). Three or four seeds of clover were also sown in each pot. *Pedicularis* seedlings grew very slowly during the first few weeks, remaining a pale green and of poor vigor, after which they began to grow more vigorously, and became a deeper green in color, apparently as a result of establishment of haustorial connections on the roots of the associated clover seedlings. Such connections were observed on washed root systems from pots of plants not used for inoculations.

A native species, *P. canadensis* L., common lousewort, also was represented by a plant from the University of Wisconsin Arboretum in Madison and several plants from the edge of a road through a forest in Oneida County, Wisconsin. These were transplanted in August to pots, along with associated soil and vegetation in which they were growing. At the same time four small plants of *P. resupinata* from Korean seed, which were growing in pasteurized soil with no other vegetation, were transplanted into some of the Arboretum soil. All plants were grown in a cold frame outside until mid-November, when they were brought into the greenhouse, after having gone into dormancy and lost their leaves. By early January new leaves were growing on both species.

Seeds of *C. miniata* came from a site approximately 50 km north of Victoria, BC. Much of the histological information on this species was obtained from examination of leaves after two different inoculations of a single plant. Subsequent inoculations of additional plants yielded supplementary information and confirmed the results seen on the first plant.

P. vulgaris 'Eagle,' a nonhost plant of the rust, was grown from seed and inoculated on the first trifoliate leaves.

Rust. The rust *C. ribicola* was maintained on plants of *R. nigrum* in the growth rooms. A mixture of aeciospores collected a few years previously from cankers on several white pine trees about 30 miles west of Madison comprised the original inoculum.

Inoculation and incubation. Inoculations were made with either freshly collected or lyophilized and refrigerated urediniospores. Urediniospores at a concentration of about 50 mg in 100 ml of 0.01% nonyl alcohol were sprayed on the undersurface of leaves. The plants were then incubated in a dew chamber for 48 or 72 hr at about 17 C. Afterwards, plants were maintained in a growth room on a 14-hr light period, a day temperature of 21 C, and a night temperature of 16 C.

Sectioning and microscopy. Small portions were cut from a leaf and fixed in 3% glutaraldehyde in phosphate buffer at pH 7.2 at desired intervals after inoculation. Some of these were previously screened for spore germination and appressorial formation by epifluorescence microscopy of leaf samples that had been treated with the fluorescent brightener, Calcofluor White M2R New (24,26). A present equivalent is Cellufluor (Polysciences, Inc. Warrington, PA.). An adequate representation of the range of growth behavior by the fungus was obtained through examination of sections from at least three samples per plant for each time period in an experiment. Dehydration was in graded ethanol-acetone followed by embedding in paraffin or Spurr's epoxy resin modified with a silicone additive to improve sectioning (19). Serial sections of resin-embedded material were made at 2-4 μm with glass Ralph knives on a standard AO Model 820 rotary microtome (American Optical Co., Buffalo, NY) modified with a specimen block retractor and a ribbon flotation collector tray (3,13). Ribbons were floated onto slides on a distilled water film, expanded at 70 C, and allowed to dry for at least 3 hr at this temperature on a slide warming table. Slides were stained in freshly prepared and filtered 0.5% Coomassie Brilliant Blue R-250 in 1.0% acetic acid for 5–15 min, rinsed in distilled water and redried at 70 C before mounting in immersion oil for examination by phase contrast microscopy. Wrinkling of the plastic matrix in and around the sections was induced by Permount mounting medium, and subsequent use of immersion oil as the mountant avoided this difficulty. Paraffin sections at a thickness of 10–12 μm were stained with safranin and aniline blue or fast green. Sections also were made from resin-embedded material that had been prestained by the technique used on whole-leaf segments. Epifluorescence microscopy was with a Leitz Ortholux microscope equipped with a Xenon XBO 150W/1 lamp, a UG-1 excitation filter, and a K430 barrier filter.

For examination of germinated spores by scanning electron microscopy (SEM), leaf samples (usually first treated with the fluorescent brightener and screened by fluorescence microscopy) were fixed in Karnovsky's fluid (equal portions of 4% formaldehyde and 4% glutaraldehyde in 0.025 M sodium phosphate buffer, pH 7.2), dehydrated in a graded ethyl alcohol series, dried by the critical-point method from amyl acetate through CO_2, and coated with gold-palladium. Specimens were examined with a Hitachi S-570 scanning electron microscope, equipped with a LaB_6 electron gun, at either 10 or 20 Kv, in the secondary electron detection mode.

Haustoria in stained sections of Spurr's resin-embedded material were selected for SEM by phase contrast microscopy. After removal of cover slips, the embedding resin was removed by soaking slides in a 3–5-day-old saturated solution of NaOH in ethyl alcohol, followed by two changes of acetone before critical-point drying (29).

Whole-leaf mounts. Observations of development of the fungus in mounts of leaf portions approximately 1-× 1-cm supplemented those made in sections. The technique for staining the fungus and clearing the leaf tissue was essentially a combination of methods described by Sargent et al (25) and Shipton and Brown (27) and by Frances Brach and L. B. Shain, University of Kentucky (*personal communication*). Leaf segments were cleared in saturated chloral hydrate solution at 95 C until they were colorless and sank. Then,

segments were transferred to lactophenol containing 0.25% aniline blue and 0.25% trypan blue at room temperature and left overnight. Excess stain was removed with saturated chloral hydrate at room temperature; destaining was accelerated if necessary by gentle warming up to about 37 C. The solution was changed several times as it became colored until stain ceased to leach from the tissue over a period of 1–3 days. Segments were rinsed and mounted in clear lactophenol. Usually, whole segments appeared blue, but under the microscope leaf tissue was more or less clear, and the rust hyphae were stained blue. The stain was retained when such tissue also was embedded and sectioned in Spurr's resin.

RESULTS

Descriptions of rust development in leaf tissue were compiled from the combined results of observations of sections and whole-leaf mounts. Most of our information came from observation of the relatively thin sections in resin. Coomassie blue tended to stain the cut walls of the fungus a lighter blue than the host tissues, and in combination with phase contrast optics a relatively good differentiation between host and fungus was almost always obtained. In the thicker paraffin sections, as well as in some embedded in glycol methacrylate, we were unable to achieve a uniformly clear differentiation between host and fungus cells and, therefore, we used the resin sections for routine use. Examination of whole-leaf mounts aided in interpretation and confirmed the impressions gained from serial sections.

On all the inoculated experimental plants, the characteristics of germination, penetration, and early stages of colonization by *C. ribicola* were essentially the same. Certain developmental features, however, were unique to growth of the fungus in *R. nigrum*: formation of haustoria, establishment of a lasting infection, and development of symptoms and signs.

Ribes nigrum. Growth of some infection hyphae stopped after penetration into the substomatal chamber (Figs. 1 and 2), so that no further development of such specimens was observed in leaf samples collected up to 16 days after inoculation. By this time uredinial and telial sori had developed from other infections. In rare instances, the infection hypha was slightly swollen in the space beneath the guard cells, but such swelling was neither as regular in form or as common in occurrence as is typical of the substomatal vesicle formed in the white pine needle (5).

Colonization occurred by ramification of hyphae in intercellular spaces of a relatively localized area between the lower and upper epidermis. By 5–6 days, hyphae may have reached the upper epidermis (Fig. 3), but in other infections hyphae were concentrated in the intercellular space of the spongy mesophyll and extended only to the bottom of the palisade tissue (Fig. 4). In most areas of colonization, hyphae were not densely packed, but ramified without severe crowding in intercellular spaces of the mesophyll (Fig. 5). In the immediate areas around uredinial or telial sori, however, hyphae were aggregated in dense masses. The mycelium did not colonize the leaf tissue uniformly, but was concentrated in localized infection centers (Fig. 6). Width of the hyphae ranged from 1 to 8 μm, but most hyphae were about 2 to 4 μm wide. The terminal cell, presumably a haustorial mother cell, of many of the hyphae often was bulbous or somewhat swollen and delimited by an evident septum (Fig. 7). A swollen terminal cell was sometimes seen at the tip of the infection hypha as early as 24 hr after inoculation, and after 3 days such cells were readily evident on hyphal branches.

Haustoria were first visible in mesophyll cells in samples collected 6 days after inoculation. At this stage the haustorium was a small globose swelling about 1 μm in diameter at the tip of a thin connecting strand.

Development of haustoria continued in tissue up to about 9 days after inoculation, beyond which time no further changes in size or form were noted. In 7-day material they were relatively common, typically globose, about 2–4 μm in diameter, and present in both mesophyll and epidermal cells. By 9 days after inoculation haustoria varied in shape from globose (Figs. 8, 9, and 14) to ovoid (Fig. 15). Most haustoria had a smooth surface, but some were irregular (Fig. 10); others were covered by a number of projections or dull spikes as on the head of a medieval mace. Some haustoria assumed a pearlike (Fig. 17) or potatolike (Fig. 11) shape and attained a size of up to 4–6 μm. The connecting neck from the haustorial mother cell was narrow and tubelike (Fig. 16); in the prestained sections it appeared only as a threadlike stained strand (Figs. 8 and 9), but in plastic sections stained with Coomassie blue, the walls of the tube were stained to outline the narrow lumen of the neck (Figs. 12 and 13). On a few sections portions of a collar were seen immediately at the inside surface of the mesophyll cell wall (Fig. 17). Often the wall of the haustorial mother cell was flattened against the wall of the host mesophyll cell (Figs. 10 and 11). As the haustorium developed it invaginated the plasma membrane of the host cell (Fig. 16) and developed in the space created between the plasma membrane and the cell wall.

Pedicularis resupinata. The early stages of penetration and colonization by the rust on this plant were similar to those on *R. nigrum*, from appressorial formation (Fig. 18) to growth in the intercellular spaces in the mesophyll. Maximum development was attained in from 3 to 6 days after inoculation, and no advance or other changes were seen in 16-day collections. The infection hypha grew as a straight or gently curving hypha through the substomatal chamber to the surface of a mesophyll cell (Figs. 19 and 20). Sometimes infection hyphae branched soon after penetration between the guard cells (Fig. 23). The furthest distance reached by growth of the infection hypha was immediately beneath the upper epidermis (Fig. 24); in this example, abortive branches had formed from the main hypha, but these did not extend beyond the course of the main infection hypha (Fig. 25). The abundant branching by the fungus and extensive colonization of the tissue that were observed in *R. nigrum* did not occur in *P. resupinata*. Often the infection hypha terminated in a bulbous haustorial mother cell similar to those seen in *R. nigrum* (Figs. 21, 22, and 24).

Pedicularis canadensis. Colonization in this species (Fig. 26) resembled that in *P. resupinata*, although in none of the sections had infection hyphae reached as far as the palisade cells.

Castilleja miniata. The behavior of the rust on *C. miniata* is summarized from observations of several series of inoculations on seven plants. In contrast to the lack of major development in *Pedicularis*, the fungus continued to grow in some plants of *C. miniata* over a period of several weeks, and colonization in portions of some leaves was extensive. The degree of colonization was variable, however, among leaf samples. In some, growth was arrested soon after penetration of an infection hypha into the substomatal chamber. In others, more growth had occurred; in some samples, collected 60 days after inoculation, mycelium was densely packed in much of the intercellular space in localized portions over 2 mm long in sections of leaf segments.

By the end of the 72-hr germination and incubation period, the infection hypha generally had reached into the mesophyll from one-half to two-thirds of the thickness of the leaf blade (Figs. 27 and 28). Often some branching of the infection hypha had occurred, but such growth was localized. Some hyphal tips became swollen and formed the typical "haustorial mother cell" seen in *R. nigrum*, but no haustoria ever developed.

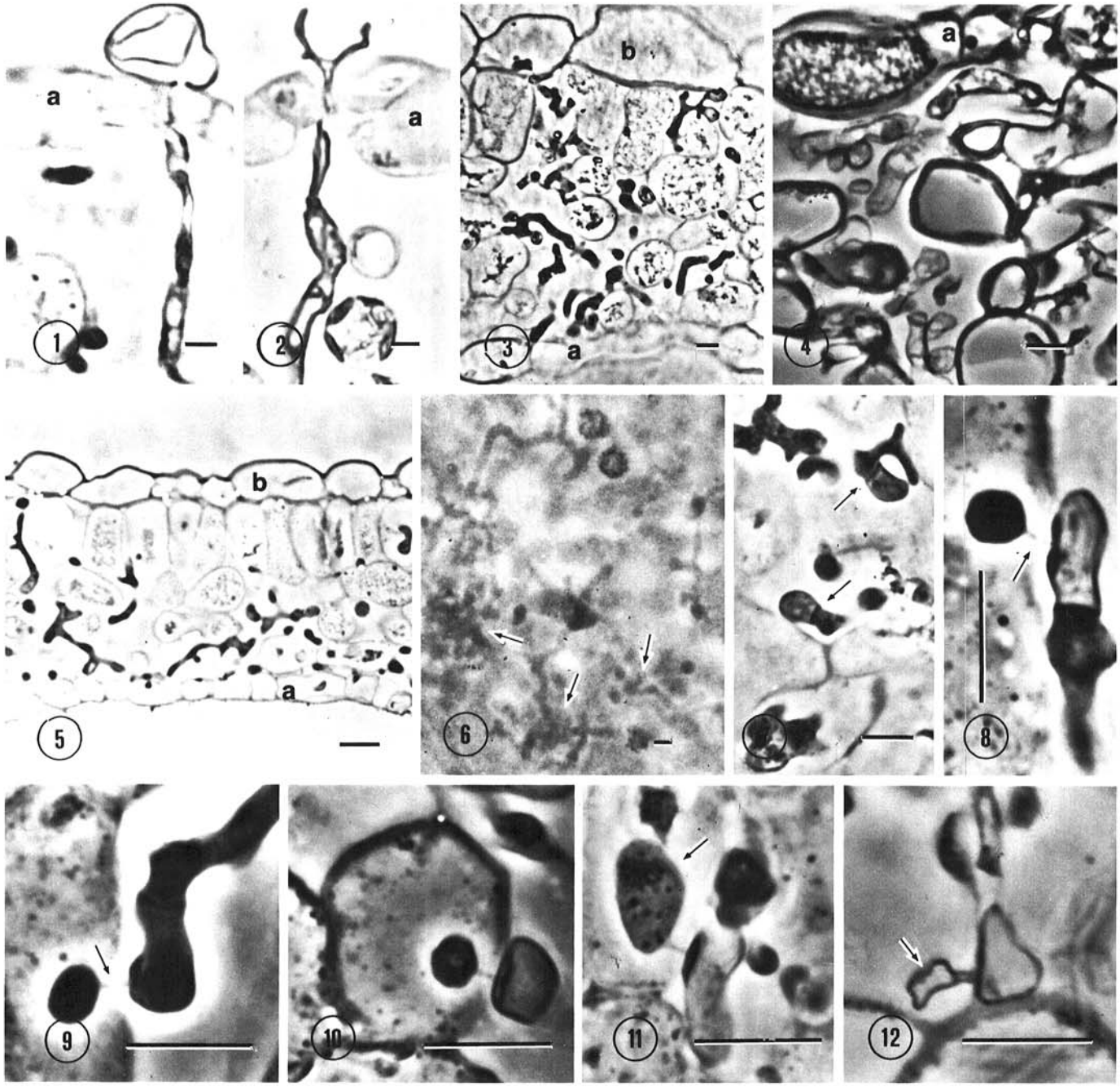
By 6 days after inoculation, branches of the infection hypha had developed (Fig. 29). The fungus had reached the upper epidermis and had extended laterally in the lower and midportions of the mesophyll. Swollen terminal cells were readily visible (Fig. 30).

In 9-day collections, colonization of leaf tissue beyond the 3- and 6-day stages was evident. The infection hypha had become much branched, and hyphal branches were curving and tortuous in their growth through adjacent intercellular spaces. Thus, they appeared in sections as irregularly shaped hyphal fragments outlined by blue-stained walls. Both lateral and vertical colonization had occurred and localized colonies had formed in intercellular spaces in the mesophyll (Fig. 31). Hyphae from such an infection center could extend laterally for as much as about 200 μm, approximately the distance covered by six to eight mesophyll cells. The localized colonies could be up to the width of three to four mesophyll cells, about 80–100 μm wide with a vertical extent of 50 μm. In leaves with this type of extensive colonization, the rust occurred in

pockets of dense concentrations of meandering hyphae. These pockets, which comprised a number of adjacent intercellular spaces, were randomly dispersed through the infected region of the leaf. In colonizing the leaf, hyphae grew with little crowding in some areas (Fig. 32) or were densely packed in others (Fig. 33).

Colonization after 16 days was similar to that in 9-day samples except that in some areas it was more extensive. In some sections dense masses of hyphae filled the substomatal chamber (Fig. 34) or localized areas of intercellular space in the mesophyll (Fig. 35).

In tangential sections such localized "islands" or colonies of tortuous hyphae (Fig. 36) appeared in the lower mesophyll tissue as irregular conically shaped masses narrowing toward the top (adaxial) surface of the leaf. None of the colonization stages showed any evidence of aggregation and differentiation of cells beneath the lower epidermis, which marked the beginning of uredinial or telial sori, as seen in *R. nigrum*. From the various degrees of colonization that were produced, we inferred that hyphae often colonized intercellular space according to the



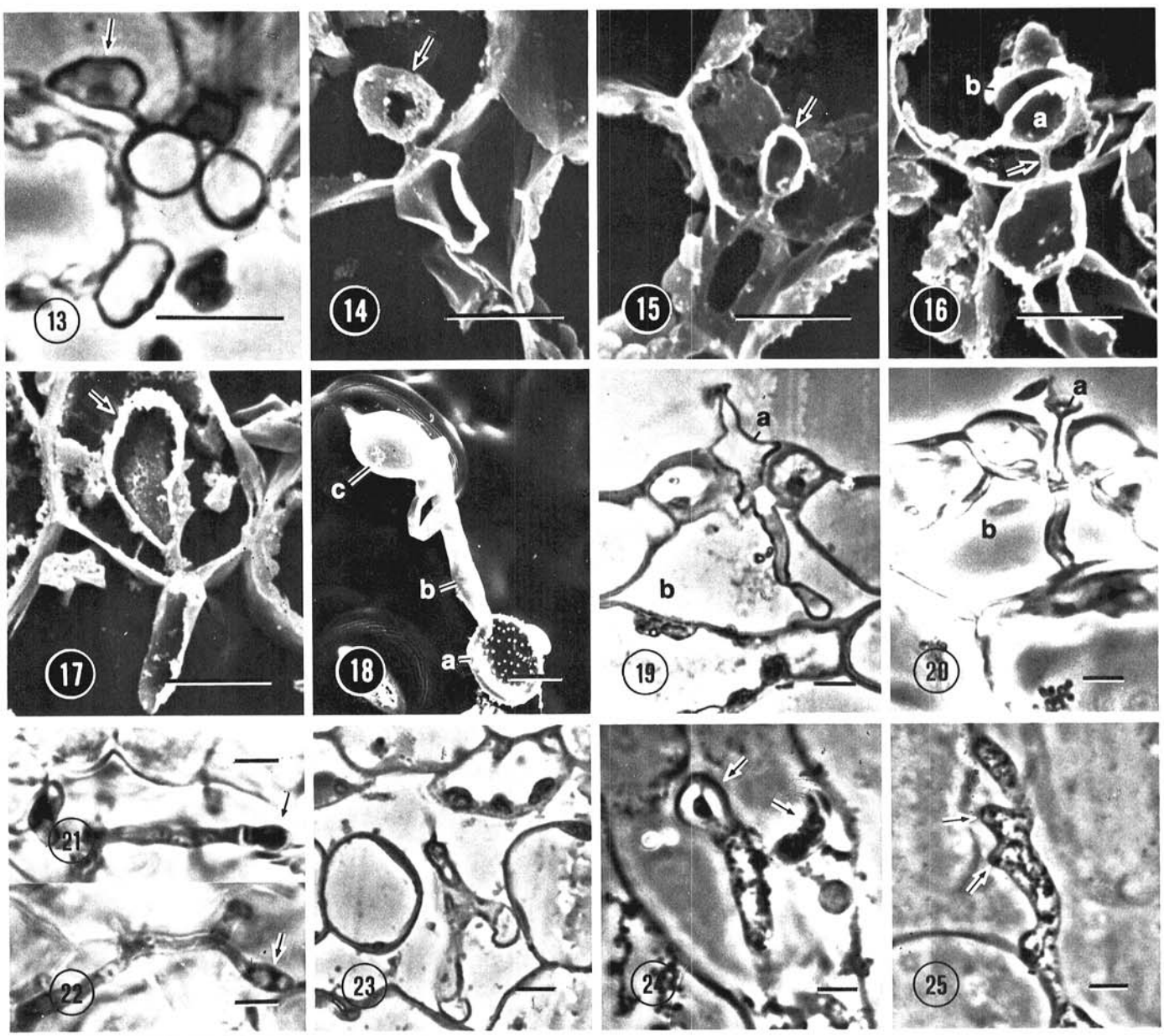
Figs. 1–12. Photomicrographs of colonization of leaf tissue of *Ribes nigrum* by *Cronartium ribicola*. **1** and **2,** Penetration of infection hypha into substomatal chamber from appressorium through stoma in lower epidermis (a). Collected 6 and 5 days, respectively, after inoculation. **3,** Colonization in localized portion of mesophyll between lower (a) and upper (b) epidermis, 6 days after inoculation. **4,** Colonization of intercellular space in spongy mesophyll above lower epidermis (a); no extension of hyphae in this area beyond bottom of palisade tissue in a sample collected 16 days after inoculation. **5,** Lateral extent of colonization from a single penetration without crowding in intercellular spaces 7 days after inoculation. Lower (a) and upper (b) epidermis. **6,** Colonization from several infection centers 2 days after inoculation as seen in a cleared whole mount. Arrows mark representative infection centers. **7,** Terminal swollen cells of hyphae, presumably haustorial mother cells, in a 6-day-old sample. Arrows indicate septum. **8** and **9,** Typical globose haustoria in palisade cells as seen in prestained leaf segments collected 9 days after inoculation. The neck appears only as a fine stained threadlike strand (arrow) which connects the haustorium with the haustorial mother cell. **10,** Haustorium in a 9-day-old sample with irregular surface connected to a haustorial mother cell with one face flattened against the wall of the mesophyll cell. **11,** Relatively large, potato-shaped haustorium (arrow), 9 days after inoculation. **12,** Irregularly shaped haustorium (arrow) connected to flattened face of the larger haustorial mother cell by the tubelike neck as seen in sections from 16-day-old samples stained by Coomassie blue. Bars = 10 μm.

opportunity afforded by the degree of interconnection among intercellular spaces. The connection among intercellular spaces may govern the lateral distance of hyphal penetration but not necessarily the density of growth. Generally, hyphae were narrower than in *R. nigrum*, ranging from 1 to 4 μm in width, and mostly about 1 to 2 μm in the densely packed masses.

On one of the larger plants the basal portion of a single leaf had persisted for several weeks after inoculation and after the distal half had been removed for earlier sampling. The upper surface of this leaf portion took on a dull yellowish or mottled bronzed appearance, and at 63 days after inoculation this leaf was harvested for sectioning. Sections were 4 mm long and covered just over half the width of the leaf blade from the edge to a point across the midvein. Throughout the entire length of the sections, areas of intensive colonization were present that occupied essentially all of the intercellular space between the upper and lower epidermis (Figs. 37 and 38). Such areas occupied a lateral distance across the leaf that ranged from approximately 300 to 800 μm. In between the areas of intensive colonization the remainder of the section was colonized to a varying extent. Thus, hyphae of *C. ribicola* ramified through all the intercellular space in this approximately 2-× 4-mm segment.

Phaseolus vulgaris. Fewer observations were made of inoculated bean specimens than of the other hosts, but the type and amount of growth of the fungus was comparable to that in *Pedicularis*, and even to that in *R. nigrum* up to about 5 days after inoculation. No symptoms developed, and no haustoria were seen.

At 2 or 3 days after inoculation the infection hypha from the



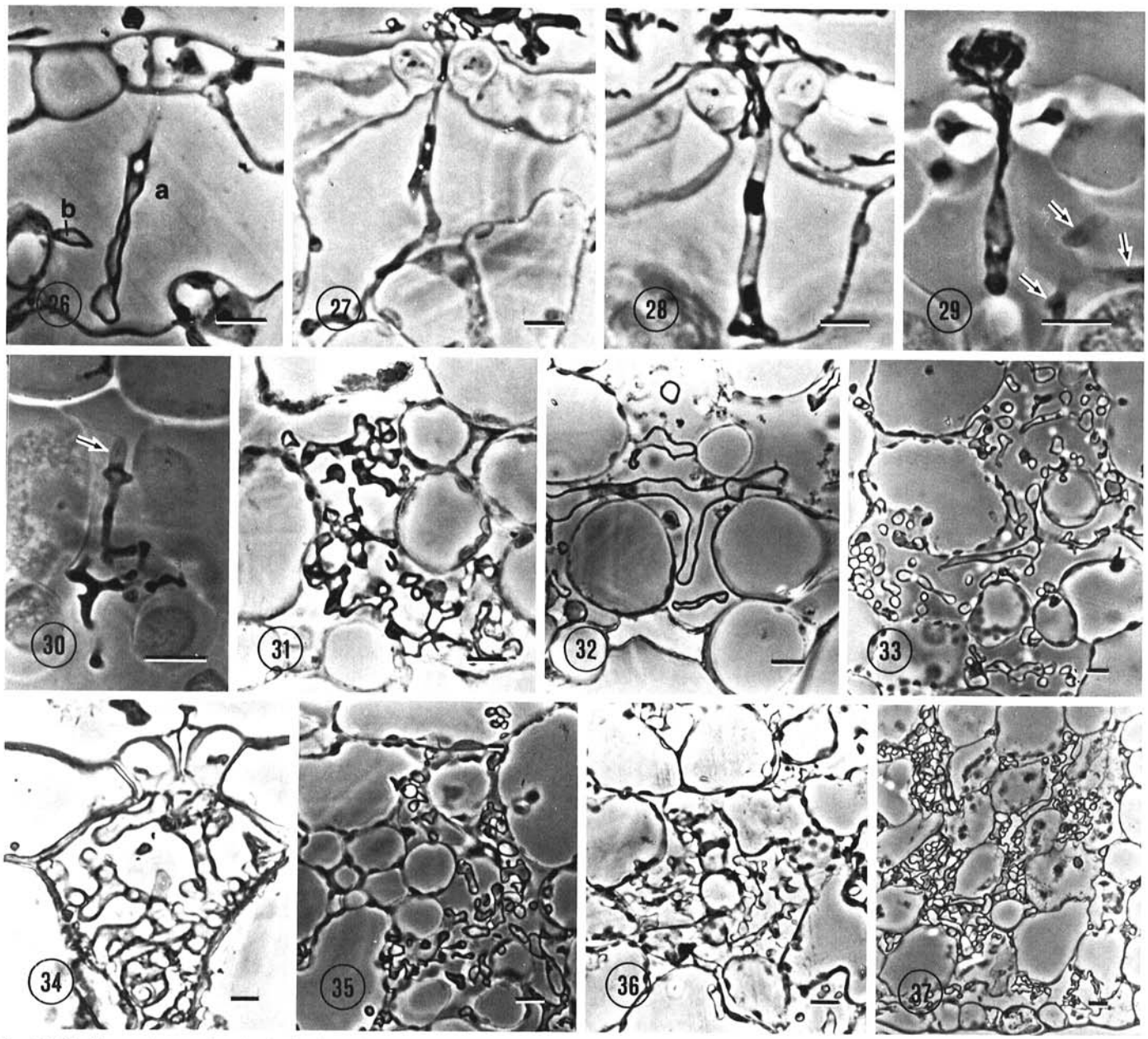
Figs. 13–25. Light and scanning electron micrographs (SEM) of colonization of *Ribes nigrum* (13–17) and *Pedicularis resupinata* (18–25) leaf tissue by *Cronartium ribicola*. **13,** Potato-shaped haustorium (arrow) in a 16-day-old sample, which is connected by a tubelike neck to a haustorial mother cell. **14–17,** SEM of haustoria in sections cut from a 16-day-old sample: **14,** Globose haustorium (arrow). **15,** Ovoid haustorium with a typical tubelike neck to haustorial mother cell. **16,** Globose haustorium (a), tubelike neck to haustorial mother cell (arrow), and invaginated portion of plasma membrane of mesophyll cell (b). **17,** Relatively large, pear-shaped haustorium (arrow) in a mesophyll cell; tubelike neck connecting to the haustorial mother cell is somewhat obscured by cell debris but was readily evident by light microscopy. **18,** SEM of urediniospore (a), germ tube (b), and appressorium (c) over stoma on underside of a leaf of *Pedicularis resupinata*. **19** and **20,** Penetration of an infection hypha into substomatal chamber (b) from appressorium (a) through stoma in lower epidermis. **21** and **22,** Infection hypha and terminal haustorial mother cells (arrows) in intercellular space just beneath lower epidermis as seen in a cleared and stained whole mount collected 8 days after inoculation. **23,** Extent of growth of branched infection hypha in substomatal chamber 16 days after inoculation. **24,** Terminal cells (arrows) of infection hypha and branch in intercellular space just beneath the upper epidermis 6 days after inoculation. **25,** Short branch initials (arrows) of infection hypha at base of palisade tissue after 6 days. Bars = 5 μm on Figures 14–17 and 10 μm on Figures 13 and 18–25.

appressorium had penetrated into the substomatal chamber and often had produced hyphal branches that extended into the mesophyll to the base of the palisade cells, to a depth of about 35 μm (Figs. 39 and 40). Segments of hyphae could be traced laterally through several sections, to a distance of about 35 μm. Branching often resulted in a Y-shaped segment at the end (Fig. 41), with the tips typically terminating in a slightly swollen "haustorial mother cell" (Figs. 42 and 43), although haustoria were not formed. At 16 days after inoculation the fungus was in this same stage of development.

DISCUSSION

Differentiation of *C. ribicola* into two formae speciales was done by Yokota and Uozumi (31) originally on the basis of differences in pathogenicity on pines after inoculations in Japan and on reports of plants that served as alternate hosts. The inability of the Wisconsin isolate of *C. ribicola* to grow intensively or reproduce in *R. resupinata* is additional evidence of a fundamental difference between the two forms, which are so similar in their morphology.

Whether *P. resupinata* is considered as a host or nonhost depends on which form of the rust is at issue. But for a given form of the rust, this consideration is a different situation from concern with pathogenicity of the rust on a susceptible or resistant variety of a host plant. Anderson (1) compared the development of *C. ribicola* in three susceptible species of *Ribes* with that in an extremely resistant host, Viking red currant. His descriptions for susceptible species were similar to our observations of rust



Figs. 26–37. Photomicrographs of colonization of *Pedicularis canadensis* (26) and *Castilleja miniata* (27–37) leaf tissue by *Cronartium ribicola*. **26,** Infection hypha (a) and segment of a branch (b) in substomatal chamber of *Pedicularis canadensis* after 5 days. **27,** Infection hypha and branch in substomatal chamber of *Castilleja miniata* 3 days after inoculation. **28,** Extent of growth of infection hypha in substomatal chamber 3 days after inoculation. **29,** Growth of infection hypha and lateral extension of branches (arrows) after 6 days. **30,** Hyphae and swollen terminal cell (haustorial mother cell?) (arrow) just beneath upper epidermis 6 days after inoculation. **31,** Localized colonization of intercellular space just above lower epidermis 9 days after inoculation. **32,** Open or loose type of growth without severe crowding or dense packing of hyphae in intercellular spaces after 16 days. **33,** Fragments of hyphae that meandered through intercellular space above the lower epidermis without crowding in a 16-day sample. **34,** Dense mass of hyphae in the substomatal chamber after 16 days. **35,** Colonization of intercellular spaces between lower and upper epidermis at 16 days. **36,** Tangential view of a localized colony of densely packed hyphae in the mesophyll after 16 days. **37,** Dense packing of hyphae in most of the intercellular space in area of intensive colonization between lower and upper epidermis in a sample 63 days after inoculation. Bars = 10 μm.

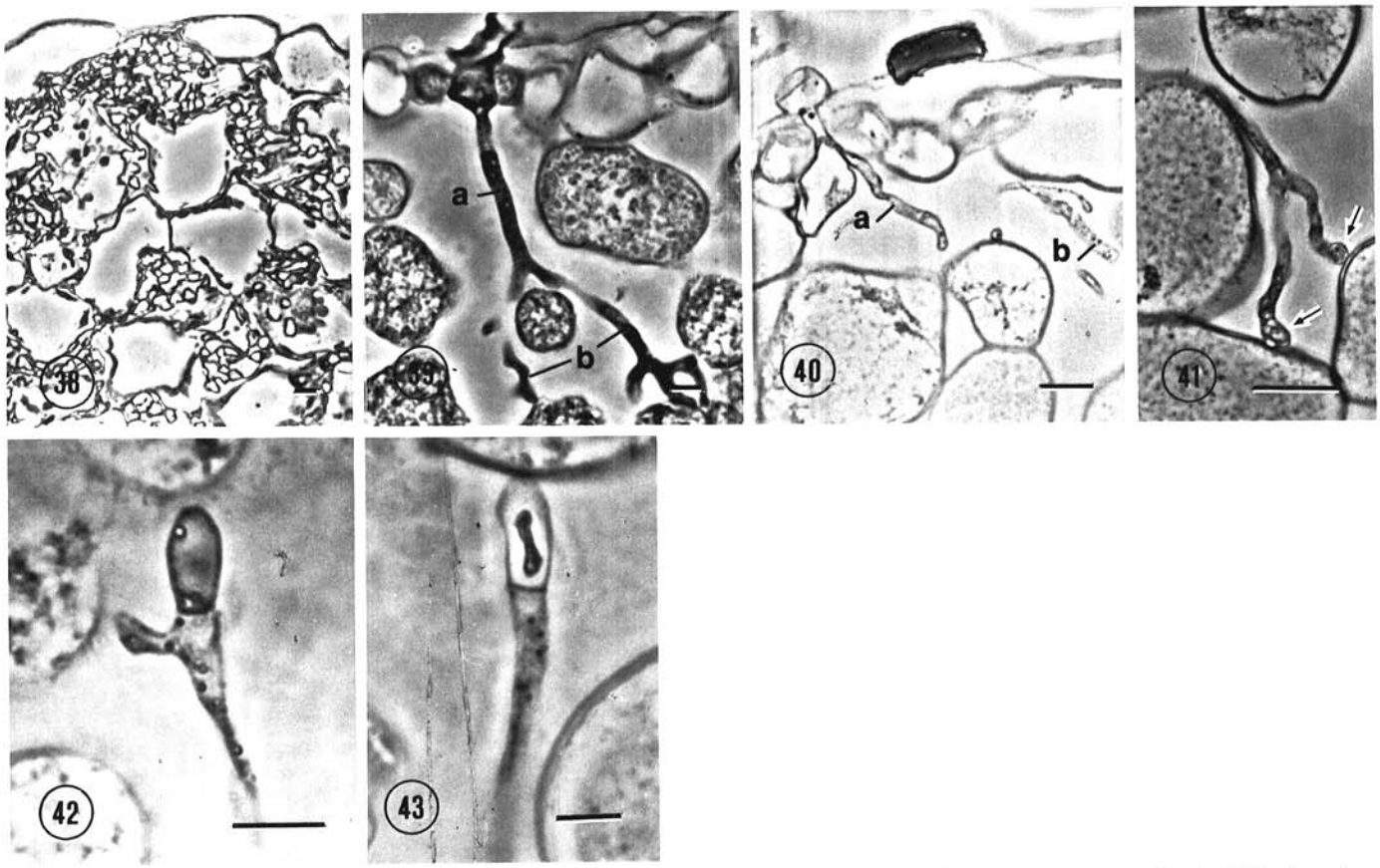
development in *R. nigrum*; the mycelium grew with profuse branching in the intercellular spaces, and little deleterious effect on host cells was noted. He did not see haustoria until infections were approximately 240 hr (10 days) old. In the resistant Viking red currant, however, penetration was the same as in susceptible hosts, but mycelium never developed extensively and within 96 hr it had broken down and disappeared. Host cells died only several days after hyphae disappeared. Thus, in this resistant host the response by the host to entry by the parasite was characterized by death and lysis of the fungus within 96 hr.

Nonhost responses, however, are fundamentally different from those expressed by resistant varieties (9). There is a lack of specificity in nonhost responses (8), and these are characterized essentially by little fungal growth beyond the infection hypha stage and an absence of haustoria (7,8,21). Such characteristics are typical of the type of growth produced by the Wisconsin strain of *C. ribicola* in *P. resupinata*, *P. canadensis*, and the common bean.

These reactions were similar to those in other rust and nonhost associations. Heath (7) found that growth of the cowpea rust beyond the formation of infection hyphae rarely occurred in nonhosts. Leath and Rowell (20) reported that *Puccinia graminis* developed no haustorial mother cells or haustoria in corn (*Zea mays*), although infection hyphae often terminated in rounded or sometimes enlarged tips. Hilu (14) described the haustorial mother cell in the compatible association of *Puccinia sorghi* in corn as an expansion of a tip of an infection hypha branch into a terminal cell marked off by a septum. This is similar to the swollen or bulbous tips of branches of the infection hypha we observed in all the species we examined; presumably these were haustorial mother cells which never developed beyond that stage, except in *R. nigrum*, a true host species.

Although host responses were not observed by light microscopy, our observations are incomplete, since we were unable to use transmission electron microscopy. The darkening effect on nonhost cell walls noted by Heath (7) was not seen. But ultrastructural signs of incompatibility were detectable by Heath and Heath (12) before certain characteristic host responses normally seen by light microscopy were observed.

The extensive colonization of leaf tissue in *C. miniata* by *C. ribicola* was surprising. Although Heath (7,8) noted that nonhost responses were characterized by little growth beyond the infection hypha stage and the absence of haustoria, in some host-parasite associations considerable fungal growth is possible before haustoria formation. Thus, as also suggested by Rice (23), such growth must result from absorption of nutrients in intercellular spaces. The earliest that we saw haustoria in *R. nigrum* was 6 days after inoculation, but they were not relatively common in occurrence until about 7 or 8 days. By this time uredinial or telial sori were beginning to form, but the bulk of leaf colonization had occurred before this hyphal aggregation and before haustoria had formed. Anderson (1) also reported that haustoria were not seen until the mycelium in the *Ribes* leaf was approximately 10 days old and uredinia had begun to develop. Also, Harvey and Grasham (6) showed that growth of mycelium of *C. ribicola* was supported by nutrients of small molecular size that diffused through cellulose membranes supporting tissue cultures even of the nonhost *Pseudotsuga menziesii* (Mirb.) Franco. Thus, the extensive growth of *C. ribicola* in *C. miniata* presumably was possible through absorption of nutrients leaking into the intercellular space. The lack of haustoria in all of our species except *R. nigrum* probably was due to the lack of appropriate stimulation from these plants, in accord with the suggestion by Heath (8) as typical of a nonhost interaction with the parasite. Such absorption may be sufficient for vegetative growth of the fungus but not for production of



Figs. 38–43. Photomicrographs of colonization of *Castilleja miniata* (38) and *Phaseolus vulgaris* (39–43) leaf tissue by *Cronartium ribicola*. **38,** Portion of an area of intensive colonization of essentially all the intercellular space in a sample of *Castilleja miniata* 63 days after inoculation. **39** and **40,** Extent of colonization of substomatal and adjacent intercellular space by infection hyphae (a) and branches (b) in samples of *Phaseolus vulgaris* 3 days after inoculation. No development beyond this stage was seen in later collections. **41,** Y-shaped branched tip of infection hypha, which terminates in haustorial mother cells (arrows) in a sample 3 days after inoculation. **42** and **43,** Terminal haustorial mother cells at tips of infection hyphae in samples 3 days after inoculation. Bars = 10 μm.

haustoria. Also, in *R. nigrum* development of uredinial (or telial) sori was concomitant with formation of haustoria. Because no haustoria or reproductive stages of the fungus were produced in *C. miniata*, even after a large amount of undifferentiated hyphal growth, perhaps haustoria are necessary for development of spore-producing structures of the fungus.

The marked difference in response to invasion by *C. ribicola* between the two *Pedicularis* species and *C. miniata* might be ascribed to the general lack of specificity of nonhost responses to rust fungi. The interactions involved in each of these systems could be regarded as unique (8,10). Another way of expressing this difference is that these species have different degrees of tolerance for this parasite. The nonhost response (or resistance mechanism?) might be the lack of haustoria formation in both *Pedicularis* and *Castilleja* species, but different sets of biochemical and physiological systems in these plants might determine the different levels of tolerance of the parasite that are expressed (4). Heath (7) suggested there are many stages during the infection process where an interaction occurs between a host plant and a parasitic fungus. Susceptibility (or a certain degree of tolerance) may depend on a "correct" response (or series of correct responses), whereas an "incorrect" response may lead to resistance (or a different level of tolerance). In any case, these nonhost responses appear to be different from the reaction by the resistant Viking currant (1) where death of hyphae at a relatively early stage was followed by lysis of hyphae and subsequently necrosis of host cells. In the nonhosts in this study, the lack of an established infection probably resulted from one or more incorrect responses, and the fungus eventually died from starvation. In *Pedicularis* species, presumably this occurred relatively soon, but in *C. miniata* the fungus continued to grow for many weeks.

Heath (11) discussed some of the theoretical problems relating to the designation of a nonhost. Perhaps bean might be regarded as a true nonhost, but the response of *P. resupinata* as a host for the East Asian strain of *C. ribicola* and the colonization of *C. miniata* by the Wisconsin strain could be considered as different levels of tolerance.

The possibility that members of the Scrophulariaceae are alternate hosts for *C. ribicola* in North America might be significant in management of the disease caused by this fungus (17). The capability of *P. resupinata* (and other *Pedicularis* species) to serve as alternate hosts for the East Asian form of *C. ribicola* probably is the result of co-evolution of host plants and fungus. As suggested by Heath (11), closely related species might be expected to have several nonhost-type defense mechanisms in common. Accordingly, the East Asian strain might require little adaptation to be able to attack other scrophulareaceous hosts such as *Pedicularis* or *Castilleja* species, if it were introduced to North America. And, similarly, perhaps association of *Castilleja* with the North American strain of the rust might result in accumulation of enough analogous mutations so that the rust could overcome the plant's nonhost resistance mechanisms and use it as an alternate host.

Our results with *C. miniata* raise further questions about its status as a host for *C. ribicola*. Hunt's (17) inoculations yielded no symptoms, but since the leaf tissue was not examined microscopically it is not known whether colonization occurred. The extensive colonization of *C. miniata* in our inoculations might be supplementary support for the infection of this species reported by Hiratsuka and Maruyama (15). Possibly the conditions in our growth chambers did not favor establishment of the rust, whereas both environmental conditions and rust isolates in Hiratsuka and Maruyama's work (15) favored further development up to formation of telia. Such conditions might induce a greater degree of tolerance of the rust, in accord with Clark's statement (4) about existence of degrees of tolerance of rust infection, particularly in wild plants. Under conditions in the West development might have continued further through the series of switching points discussed by Heath (7). Or if the isolates used by Hiratsuka and Maruyama (15) represented the East Asian form of *C. ribicola*, this form being pathogenic on *P. resupinata*, it might also be so on *C. miniata*. The presence of this form in North America could have serious implications for the breeding program for white pine blister rust resistance. Another view is that tolerance of the North American form of the rust by *C. miniata* (and other *Castilleja* species?) has developed to a point so that sporulation from natural infection by the rust occurs on *Castilleja* plants in the wild. Presumably spores seen on *Castilleja* in the past could have been taken for those of *Cronartium coleosporioides* Arth. If such a situation existed, white pine blister rust control strategies could be affected.

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