Invasion Mechanisms Of Cronartium ribicola in Pinus monticola Bark

Bruce L. Welch and Neil E. Martin

Biological Laboratory Technician and Research Plant Pathologist, U.S. Department of Agriculture, Forest Service, Intermountain Forest and Range Experiment Station, Ogden, Utah 84401; stationed in Logan, Utah, and Moscow, Idaho, respectively.

Accepted for publication 19 July 1974.

ABSTRACT

In *Pinus monticola* bark infected with *Cronartium ribicola*, the main mode of pathogen invasion in the peripheral and midcanker areas of the rust canker is by "passive" growth through host intercellular spaces; there was no evidence of mechanical force being involved. Scanning electron microscopy of *P. monticola* bark revealed numerous large intercellular spaces in the cortical parenchyma. Intercellular space accounted for about 30% of bark volume. Evidence is provided of pectinase activity and mechanical force in the total invasion of the aecial area. Scanning electron microscopy also revealed that *C. ribicola* hyphae are frequently affixed to host cell walls. An hypothesis concerning the physiological significance of this phenomenon is discussed.

Phytopathology 64:1541-1546

Additional key words: intercellular space, host-pathogen interaction.

A few workers (1, 4, 7, 10, 11, 14) have examined white pine bark infected with the causative organism of the blister rust disease, *Cronartium ribicola* J. C. Fisch. ex Rabenh. They have described the effects of this pathogen on host cells and the initiation, growth, and development of various pathogenic structures. However, none have addressed themselves to how *C. ribicola* invades or spreads through *Pinus monticola* Dougl. bark. Therefore, the intent of this study was to investigate, by means of scanning electron microscopy (SEM), the way in which *C. ribicola* invades or spreads through *P. monticola* bark.

In an earlier SEM study of *P. monticola* bark infected with *C. ribicola*, we noted some internal relationships of the pathogen to its host (12). Because of that preliminary study, we decided that SEM would make possible the kinds of observations needed to determine how the pathogen invades *P. monticola* bark. Scanning electron microscopy offers (i) higher degree of resolution together with a greater depth of field (× 100, 1,000μm) than light and transmission electron microscopy (5, 6); (ii) ease of tissue or sample preparation (5, 6, 12); (iii) preservation of delicate botanical specimens by critical-point drying (2, 3); (iv) stereo pairs of micrographs; and (v) a large quantity of sample area (15- by 1- to 2-mm longitudinal section of bark) that can be examined during a given time period.

MATERIALS AND METHODS.—Five rust-infected *P. monticola* pines, each containing a single bole canker, were selected from 7- to 8-year-old nursery stock to furnish material needed for this study.

Noninvaded bark was excised from trees 10.2 cm (4 inches) above the yellow discoloration of the canker to avoid mycelia (7).

Invaded bark was excised from three different zones along the five bole cankers (Fig. 1). Zones are defined as follows: (a) the peripheral area, all bark tissue from 5 mm within to 10 mm beyond the yellow discoloration of the canker; (b) the midcanker area, all bark tissue from the peripheral area to within 7 mm of the aecia; (c) the aecial area, all bark tissue from midcanker area to 20 mm into the aecia-producing zone (Fig. 1). All bark samples were cut to the wood and into longitudinal sections (15 to 20 mm).

Treatment 1.—Bark samples from trees 1, 2, and 4 were placed in the following solutions for 10 min: 0.2 M phosphate buffer (pH 7.3 to 7.4), and a dehydration series of 30%, 50%, 70%, and 100% ethanol, and then a series of 25%, 50%, 70%, and 100% Freon TF (diluent was absolute ethanol). Bark samples were stored in 100%

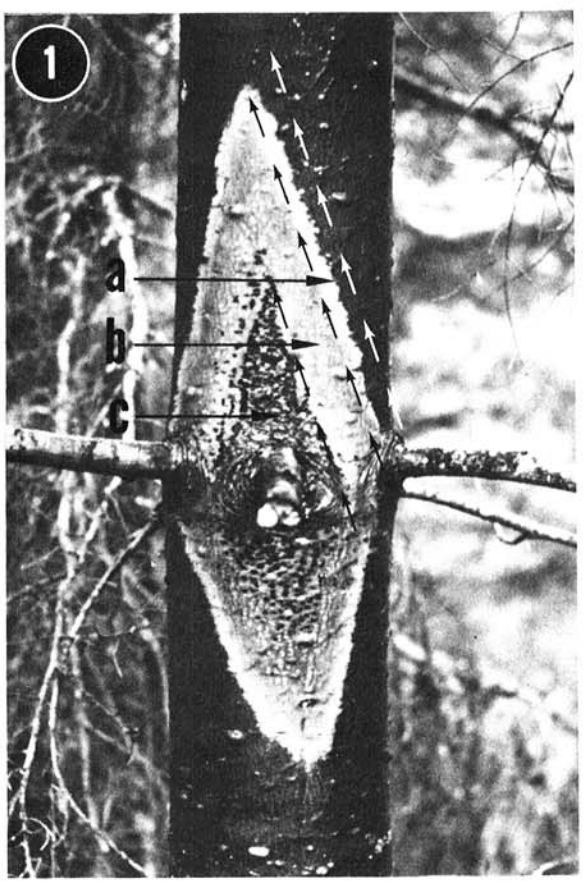


Fig. 1. A blister rust canker on *Pinus monticola*. Arrows outline (a) the peripheral, (b) midcanker, and (c) aecial areas.

Freon TF until they were critical-point dried (2, 3). Then, they were cemented to specimen holders with conductive silver paint and coated in a vacuum evaporator with a thin (20 to 30 nm) layer of gold. After coating, the samples were ready for examination by means of scanning electron microscopy.

Treatment 2.– Tree 5 bark samples were first dehydrated as described, then held in 100% ethanol for 24 h, and finally rehydrated by reversing the ethanol series to 100% glass-distilled water. Half of the bark samples were placed in a buffered (pH 5.0, 0.1 M citric acid-sodium citrate) 10% solution of pectinase (purified grade from

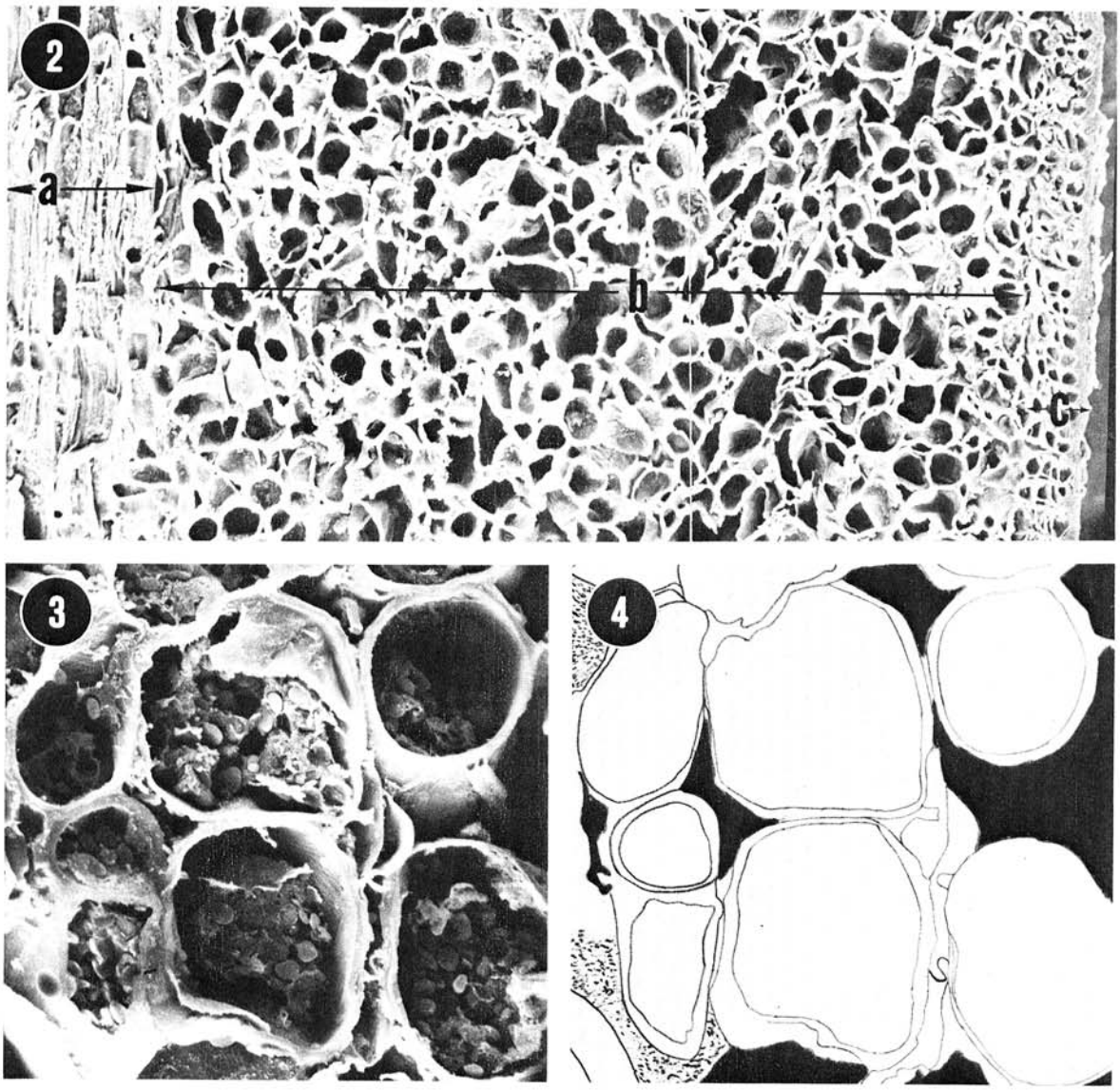
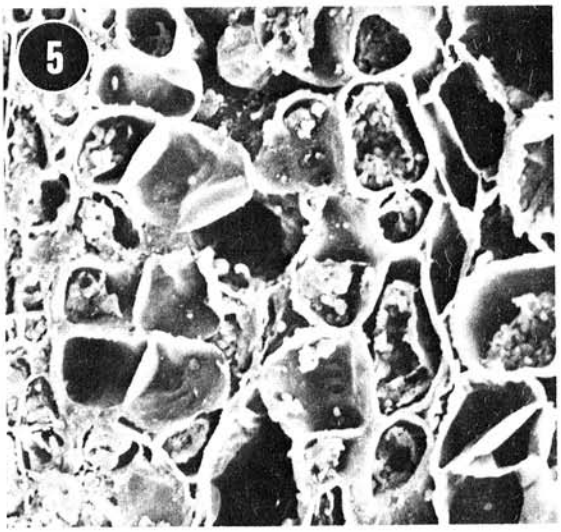
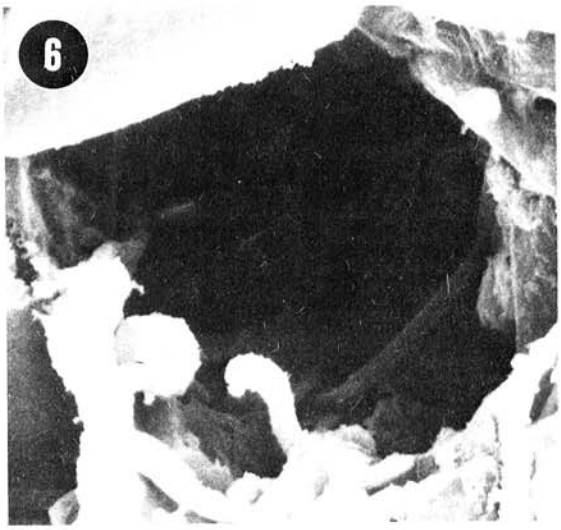
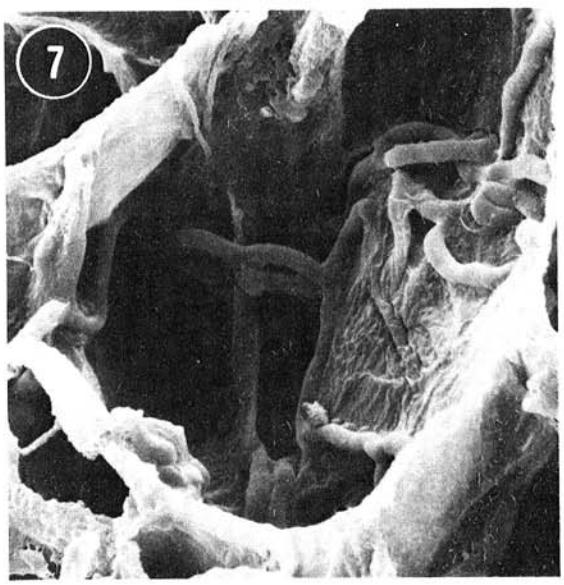


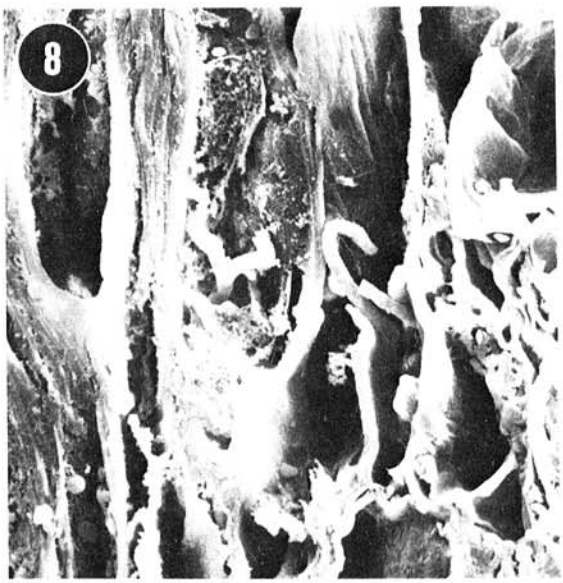
Fig. 2-4. Scanning electron micrographs of *Pinus monticola* bark infected with *Cronartium ribicola* (longitudinal sections). **2)** A composite showing the three tissues comprising the bark: (A) phloem, (B) cortical parenchyma, and (C) periderm [arrow points to invading hyphae, peripheral area, treatment 2 (× 131)]. **3)** Hyphae affixed to host cell wall, midcanker area, treatment 2 (× 163). **4)** An overlay drawing of Fig. 3. Obvious intercellular spaces have been darkened and less obvious spaces speckled.

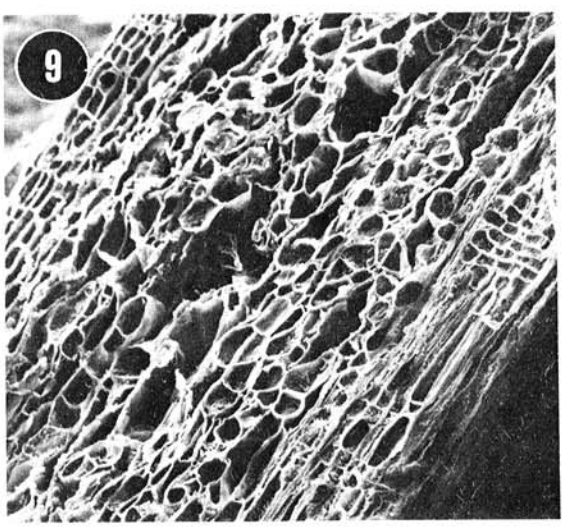
Fig. 5-10. Scanning electron micrographs of *Pinus monticola* bark infected with *Cronartium ribicola* (longitudinal sections). **5)** Hyphae confined to a large intercellular space in cortical parenchyma tissue, midcanker area, treatment 2 (× 296). **6)** A closeup of the intercellular space in Fig. 5 (× 1,257). **7)** A micrograph of an intercellular space in cortical parenchyma showing several hyphae affixed to host cell walls, peripheral area, treatment 1 (× 1,257). **8)** A micrograph of phloem showing hyphae in the limited intercellular spaces of this tissue, midcanker area treatment 1 (× 566). **9)** A micrograph of a total longitudinal section of bark showing large intercellular spaces, peripheral area, treatment 1 (× 85.5). **10)** A closeup of one of the intercellular spaces in Fig. 9 (× 350).

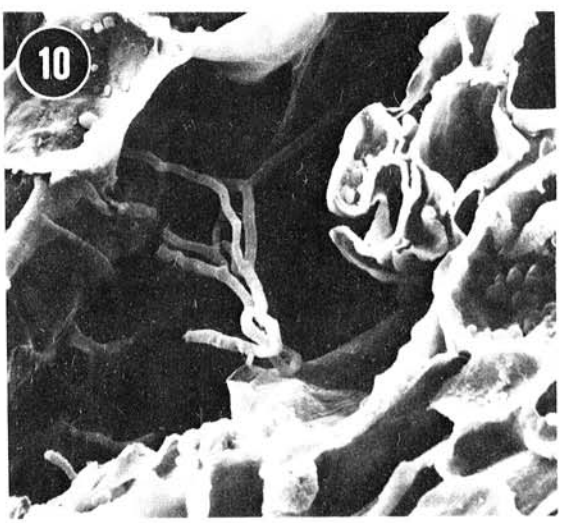

5

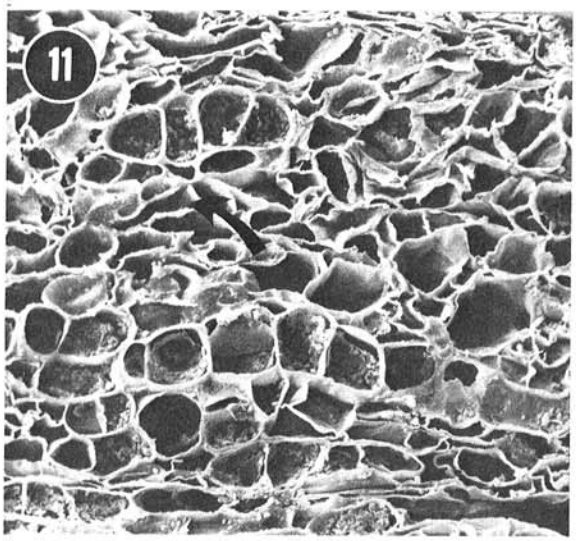

6

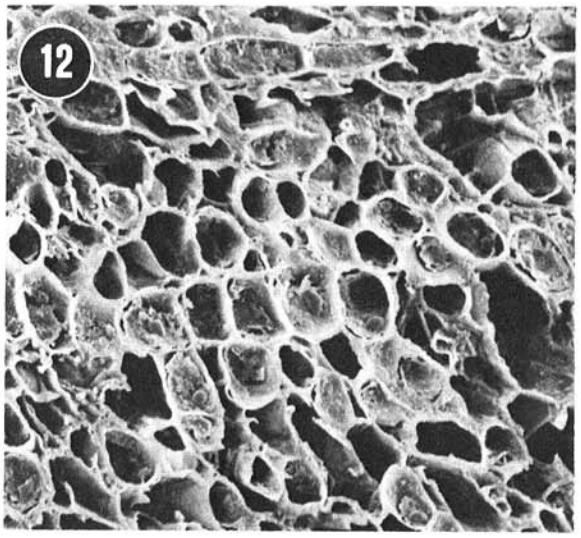

7

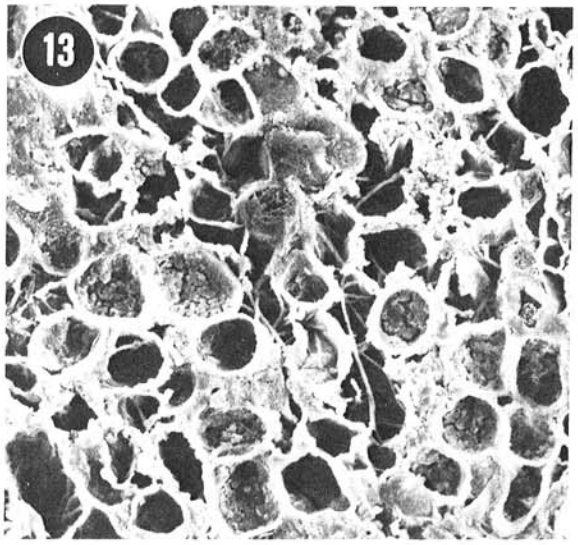

8

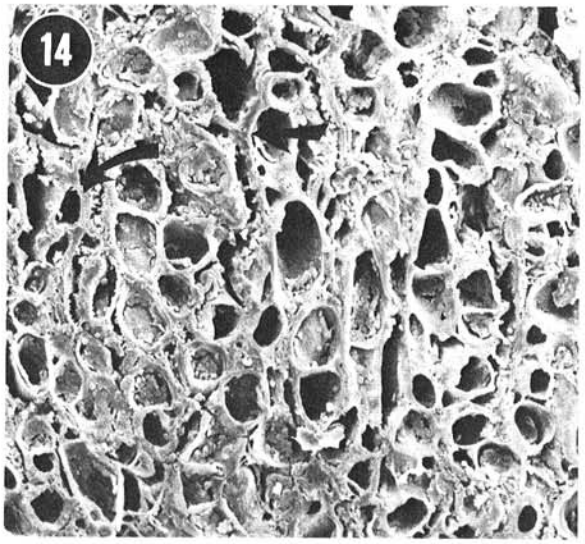

9

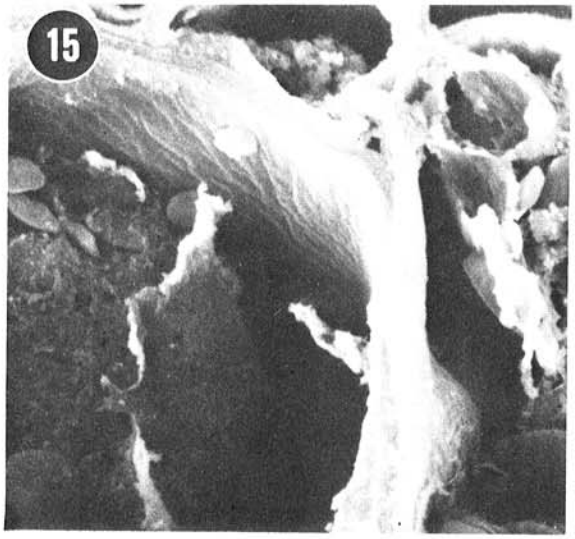

10

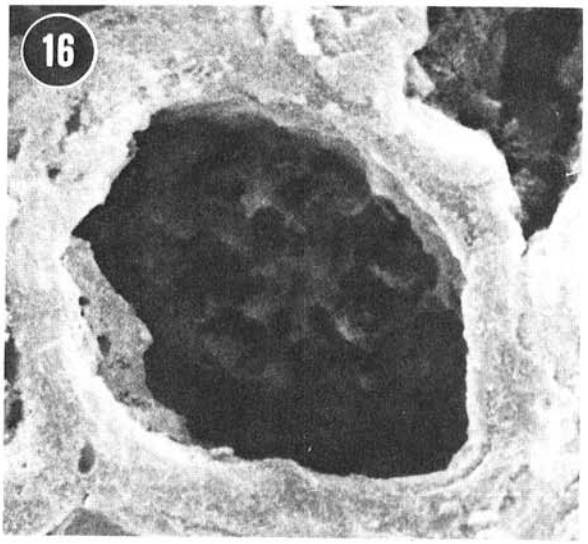

11


12


13


14


15


16

Sigma Chemical Co., 1 unit per mg) for 48 h at room temperature. The remaining samples were held in the buffer solution without pectinase. After this treatment, all samples were processed through the ethanol and Freon series outlined previously.

Treatment 3.—Tree 3 bark samples were pre-fixed for 90 minutes in 0.1 M phosphate buffer (pH 7.3 to 7.4) containing 3% glutaraldehyde. Then, they were rinsed three times (5 minutes each time) in the 0.2 M phosphate buffer and fixed for 60 minutes in 1.0% osmium dissolved in 0.1 M phosphate buffer (pH 7.4). After osmium fixation the samples were rinsed three times in phosphate buffer, dehydrated, critical-point dried, and coated with gold as previously described.

Intercellular space was quantified by weighing the scanning micrograph of a bark section, cutting out obvious intercellular spaces, and reweighing the micrograph. The difference was divided by the total weight of the micrograph and the quotient multiplied by 100 to obtain percent of intercellular space. Stereo pairs of scanning electron micrographs were used as an aid in identifying intercellular spaces in the seven (three noninvaded, four invaded) bark sections used in this study.

RESULTS AND DISCUSSION.—There were no observable differences among the treatments, except for cell wall swelling noted in the pectinase treatment.

The three tissues (phloem, cortical parenchyma, and periderm) which comprise the bark of *P. monticola* can be easily differentiated in longitudinal section by SEM (Fig. 2). The most striking feature of bark is the quantity of intercellular space (Fig. 3) in the cortical parenchyma that, at times, makes this tissue very porous (Fig. 5 and 9). Figure 4 is an overlay drawing of Fig. 3; obvious intercellular spaces have been marked.

The dark areas of Fig. 4 represent portions removed in determining percent of intercellular space. In the seven micrographs examined for this study, percent of intercellular space varied from 14% to 38% (mean, 28%). No differences between noninvaded and invaded bark were noted. In all sections, there were areas that could not be identified either as cells or as intercellular spaces, even with the aid of stereo optics (Fig. 5). These areas had the appearance of intercellular spaces; i.e., they lacked cellular contents and enclosing walls (bottoms), but were surrounded by cell wall material. We believe that these areas are actually intercellular spaces surrounded by the remains of cells that died during the course of bark development and growth. Because these questionable areas were not included as intercellular space, the figures given probably represent conservative estimates of the real percentages, which could be as high as 40% to 50% bark volume. Therefore, we conclude that enough space exists in bark to allow the pathogen to invade the pine host bark by passive growth through these spaces.

Figure 2 reveals the nature of initial fungus invasion (peripheral area). The only evidence of *C. ribicola* invading this section is a small clump of hyphae in a large intercellular space of the cortical parenchyma. In contrast, others have stated that the pathogen advances in the most recent phloem parenchyma (4) or in the phelloderm (10).

Higher magnifications revealed that all the hyphae in the intercellular space pictured in Fig. 2 were affixed to host cell walls [see Fig. 3, 6, 7, and literature citation (12)]. This observation contrasts with that of another report (10), which stated that invading hyphae were growing loosely among host cells. We have also noted hyphae unattached to host walls (Fig. 8, 9, 10, 13). In addition, we have observed numerous hyphae affixed to host cell walls in all portions of the bole cankers studied. We have concluded that *C. ribicola* hyphae can be affixed to host cell walls or can bridge intercellular spaces (12), and we hypothesize that the pathogen obtains nutrients from the host in areas where hyphae are affixed to host cell walls. That hypothesis is based on the following: (i) the growth of vegetative hyphae on membranes placed above noninfected pine tissue cultures (8); (ii) the culturing of vegetative hyphae on a medium consisting of mineral salts, glucose, agar, and a sulfur amino acid (9); and (iii) the absence of haustoria in the peripheral area of bole cankers (12). We believe that the above observations indicate that *C. ribicola* can maintain vegetative growth from a simple array of nutrients provided by the exudates from host cells. It is also possible that the rust could induce exudation through changes in membrane permeability where hyphae are affixed to host cell walls.

Invasion of host tissue by *C. ribicola* from an established infection center and sequential canker development is a three-stage process. During the formation of the peripheral area, the invading hyphae grow passively through the intercellular spaces of the host and beyond the yellow discoloration for a distance of 1.5 to 2.3 cm (7, 11). Here, the pathogen could conceivably obtain its nutrients from the cellular exudates of the host or by altering the permeability of the host membranes. Formation of the midcanker area is accomplished when the pathogen ramifies from the initial invading hyphae and spreads throughout the bark. The chief invasion mechanism in the midcanker is passive growth through intercellular spaces.

During the final stage of blister rust canker development (aecial area), haustoria have penetrated numerous host cells and great masses of hyphae have developed in intercellular spaces (10, 11, 14). The mass of pathogenic tissue is so great in intercellular space that host cells are split apart (1, 4, 14). Mechanical force does play an important role in the invasion and rupturing processes of the aecial area.

The results of the pectinase experiment are shown in

Fig. 11-16. Fig. 11-14 is a series of scanning electron micrographs showing accumulation of host substances. **11)** Noninvaded *Pinus monticola* bark; arrow points to a less obvious intercellular space, treatment 1 (× 169). **12)** Peripheral canker area, treatment 3 (× 196). **13)** Midcanker area. Note hyphae growing loosely among host cells, treatment 3 (× 201). **14)** Aecial area; arrows point to swollen cell walls, treatment 1 (× 201). Fig. 15 and 16 are scanning electron micrographs of *Pinus monticola* bark infected with *Cronartium ribicola* (longitudinal sections). **15)** A micrograph of host cell wall not treated with pectinase, midcanker area, treatment 2, (× 1,710). **16)** A micrograph of host cell wall treated with commercial pectinase, midcanker area, treatment 2 (× 1,690).

Fig. 15 and 16. Figure 15 represents the samples from tree 5 that were not treated with pectinase. The smoothness of cell walls compares favorably with that observed in cell wall sections of other treatments; it contrasts with the swollen condition of pectinase-treated samples (Fig. 16). Swollen walls in the aecial area resembled walls swollen as a result of treatment with pectinase, suggesting that pectinase was involved in the fungal invasion and rupturing processes. This is in agreement with our report of a significant 44% decrease of extractable pectic substances from infected bark (13).

During the production of pycnia, pycnial fluid, and pycniospores which occurs in the midcanker area, the host appears to react to pathogen's increased activity and to the formation of haustoria (4, 10) by synthesizing substances that accumulate lightly in the peripheral area to a heavy accumulation in the aecial area. Figures 11, 12, 13, and 14 show this gradual buildup of host substances (probably tannins, or polyphenols) from the peripheral area (Fig. 12); to the midcanker area (Fig. 13); through the aecial area (Fig. 14). Figure 11 shows the absence of such host material in noninvaded bark. This agrees with our report of a significant increase of 95%-ethanol and petroleum ether-soluble substances in infected bark (13). Others have reported observing "a yellow-brown ergastic substance" in infected bark (1, 14). Unfortunately, this material is so dense in the midcanker and aecial areas that many haustoria are not observable by SEM. Studies need to be undertaken to determine the frequency of haustoria in the three canker zones.

LITERATURE CITED

1. BOYER, M. G., and P. K. ISAAC. 1964. Some observations on white pine blister rust as compared by light and electron microscopy. Can. J. Bot. 42:1305-1309.
2. COHEN, A. L., and G. E. GARNER. 1971. Delicate botanical specimens preserved for scanning electron microscopy by critical point drying. Pages 450-451 *in* Proc. 29th Annu. Meet. Electron Microsc. Soc. Am. (Abstr.).
3. COHEN, A. L., D. P. MARLOW, and G. E. GARNER. 1968. A rapid critical point method using flurocarbons ("Freons") as intermediate and transitional fluids. J. Microsc. 7:331-342.
4. COLLEY, R. H. 1918. Parasitism, morphology and cytology of Cronartium ribicola. J. Agric. Res. 15:619-659.
5. CORLETT, M. 1970. Studying fungi with the scanning electron microscope. Greenhouse-Garden-Grass 9:1-5.
6. CORLETT, M. 1970. Surface structure of the urediniospores of Puccinia coronata f. sp. avenae. Can. J. Bot. 48:2159-2161.
7. EHRLICH, J., and R. S. OPIE. 1940. Mycelial extent beyond blister rust cankers on Pinus monticola. Phytopathology 30:611-620.
8. HARVEY, A. E., and J. L. GRASHAM. 1970. Growth of Cronartium ribicola in the absence of physical contact with its host. Can. J. Bot. 48:71-73.
9. HARVEY, A. E., and J. L. GRASHAM. 1974. Axenic culture of the mononucleate stage of Cronartium ribicola. Phytopathology 64:1028-1035.
10. HIRT, R. R. 1964. Cronartium ribicola, its growth and reproduction in tissues of eastern white pine P. 5-30 *in* N. Y. State Coll. For. Syracuse Univ. Tech. Publ. 86.
11. KREBILL, R. G. 1968. Histology of canker rusts in pines. Phytopathology 58:155-164.
12. WELCH, B. L., and N. E. MARTIN. 1973. Scanning electron microscopy of Pinus monticola bark infected with Cronartium ribicola. Phytopathology 63:1420-1422.
13. WELCH, B. L., and N. E. MARTIN. 1974. Evidence of pectinase activity between Cronartium ribicola and Pinus monticola. Phytopathology 64:1287-1289.
14. WICKER, E. F., and J. Y. WOO. 1973. Histology of blister rust cankers parasitized by Tuberculina maxima. Phytopathol. Z. 76:356-366.