

## Cell Necrosis and Fungus Content in Fusiform Rust-Infected Loblolly, Longleaf, and Slash Pine Seedlings

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Mention of trade names is solely to identify material used.

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### ABSTRACT

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Galled seedlings of loblolly, longleaf, and slash pine contained necrotic areas that were positive for tannin. These areas contained rust hyphae that exhibited peroxidase activity. Cell necrosis and deposition of tannin in infected

tissues of slash pine appeared to be a normal host reaction to the pathogen. Tannin accumulation does not seem to be responsible for fusiform rust resistance since it normally occurs in actively growing galls.

*Additional key words:* *Cronartium quercuum* f. sp. *fusiforme*, tannin, peroxidase, *Pinus*, resistance.

The role of cell necrosis and tannin accumulation in fusiform rust disease caused by *Cronartium quercuum* (Berk.) Miy. ex Shirai f. sp. *fusiforme* (*C. fusiforme* Cumm.) (4) has been explored by Jewell and Speirs (11) and Miller et al (16). These processes are thought to be part of a resistance mechanism in slash pine (*Pinus elliotii* var. *elliottii* Engelm.). If this were so, necrotic tissues could be used as a resistance marker, as they are in white pine blister rust (9, 13). In slash pines, necrotic areas are identified easily on needles and stems (20), and tannin deposits are seen readily in transverse cuts (11, 16).

Cell necrosis and tannin accumulation probably do not indicate resistance of southern pines to fusiform rust. Large quantities of dead cortical tissue appear during periods of active hyphal growth such as during development of aeciospores and pycniospores (12). Also, necrosis and tannin accumulation are accelerated when cultured cells from susceptible slash pine are inoculated with rust fungus (*R. C. Hare, personal communication*). It is generally recognized that purple spots, which are areas of cell necrosis, occur on needles and stems of both susceptible and resistant seedlings (20). However, Miller et al (16) described two types of spots as hypersensitive reactions to the fungus on stem tissues.

I have examined fusiform rust infections to determine whether cell necrosis and tannin deposition are normal responses to the pathogen or indicators of resistance. Actively developing galls were examined on pines inoculated with rust fungus cultures. Cell necrosis and tannin deposition were measured in relationship to the amount of tissue invaded by the fungus.

### MATERIALS AND METHODS

Galls from fusiform rust-infected loblolly (*P. taeda* L.), longleaf (*P. palustris* Mill.), and slash pine seedlings were

examined for tannins, cell necrosis, and fungal hyphae. Half-sib families (offspring with one common parent) of all three pine species were grown at Gulfport, Mississippi, and inoculated according to the procedure of Snow et al (19). Additional families of loblolly and slash pine, which were inoculated as described by Laird and Phelps (14), were obtained from the Forest Tree Resistance Facility at Bent Creek, North Carolina. A total of 12 half-sib slash pine families were used: two had high field resistance, five had moderate field resistance, and five were susceptible. Eleven half-sib loblolly pine families were examined: two were resistant, six were intermediate, and three were susceptible. Only two bulk seed lots of longleaf pine were inoculated.

All pine seedlings were 6-8 wk old when inoculated. Infected tissues of slash pine were examined microscopically at 3-60 days and 7, 9, and 12 mo after inoculation. Loblolly pine tissue was examined after 9 mo and longleaf tissue after 7 mo.

Galled stem tissues were stripped of needles, cut into segments 3-5 mm long, and examined for discolored areas that indicate tannin and cell necrosis. Sections of paraffin-embedded and fresh specimens were observed microscopically. Specimens were fixed in formalin acetic acid alcohol (2), dehydrated, and embedded as described by Hall et al (7). Sections from embedded samples were cut with a rotary microtome at 8-12  $\mu$ m thickness; sections from fresh specimens were cut 30-50  $\mu$ m with an Oxford Vibratome. Sections were stained according to the methods described by Czabator (*unpublished* [Final Report, Study FS-SO-2208-20.27, Southern Forest Experiment Station, Gulfport, MS 39503]), Hall et al (7), or Jewell et al (12). Tannin and tannin-precursors were identified as described by Hall et al (7) and Mace and Howell (15). Peroxidase activity, which was used as an index of viability of the rust fungus, was determined by the technique of Gagnon (6) except that tetramethylbenzidine was used instead of benzidine in some of the

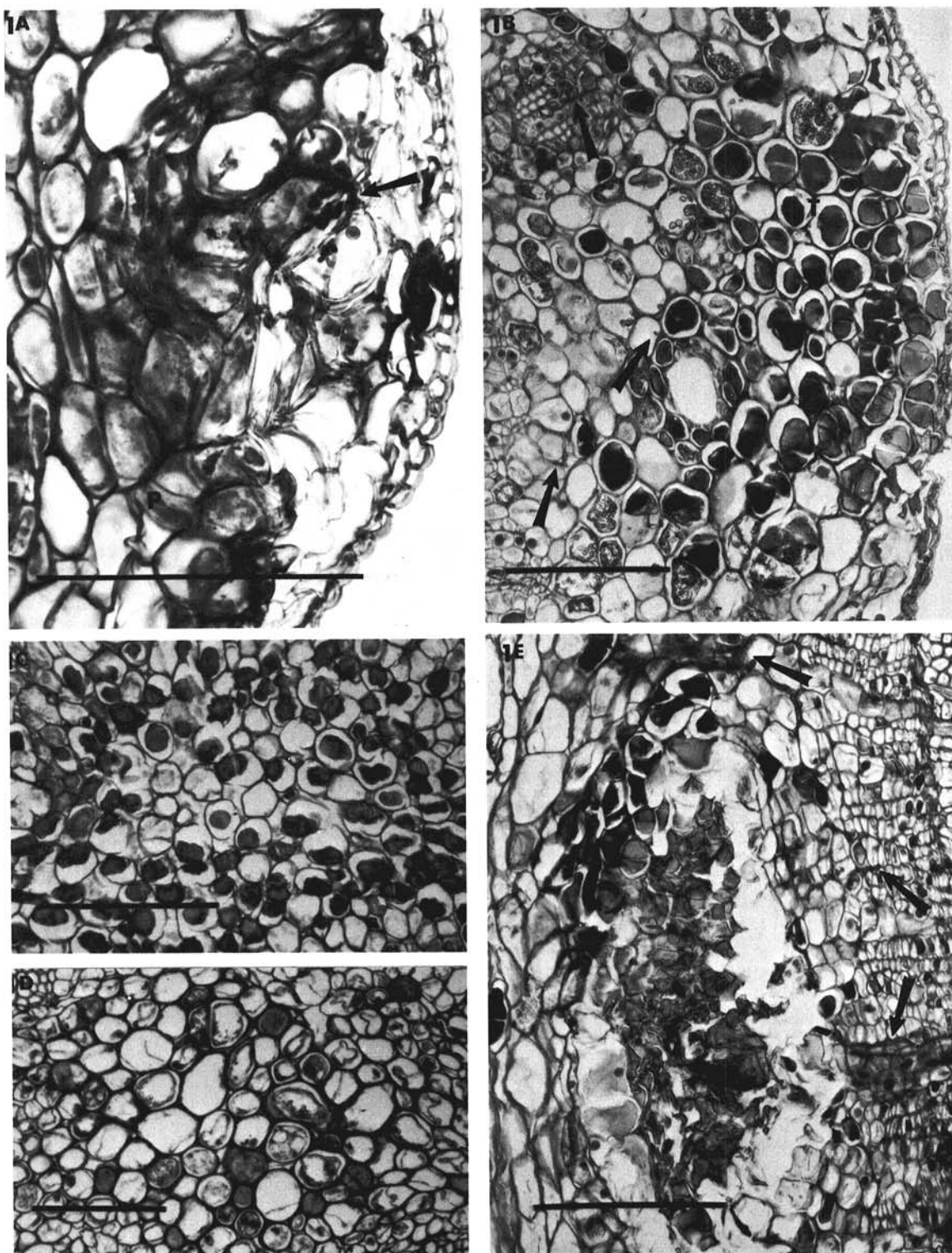


Fig. 1—(A to E). Necrotic areas in slash pine seedlings 90 days after inoculation with the fusiform rust fungus, *Cronartium quercuum*. A) Necrosis with periderm (P) and peroxidase-positive hyphae (arrow). B) Zone of necrosis and tannin-filled cells (T). Fungal hyphae (arrows) are in needle trace and near cambium. C and D) Early stages of necrosis of pith cells. E) Area of necrosis in cortex. Hyphae are visible at several points (arrows). Scale bars represent 200  $\mu$ m.

assays. Area measurements of infected tissues were made with an ocular micrometer, and photomicrographs were taken with a Carl Zeiss Photomicroscope II.

### RESULTS

A high content of tannin accompanied cell necrosis in young and older pine seedlings that contained living hyphae (peroxidase positive). The necrotic areas occurred in the cortex, xylem, and pith. They ranged from as few as 10 cells to more than one-half the cross-sectional area of the gall.

The presence of tannin did not limit the development of rust hyphae. Tannin deposition could be seen, however, in areas where the fungus was restricted by cellular barriers. One example was in young slash pine seedlings. The necrosis extended inward from the epidermis of the needle base and was bordered by a well-developed periderm (Fig. 1-A). In transverse sections, the necrotic area contained about 0.11 mm<sup>2</sup> of dying and tannin-filled parenchymal cells. Such necrotic areas were detected microscopically by 9-12 days and the periderm was developed by 19 days after inoculation. These areas occurred at the site of inoculation and could be seen in transverse sections. Normally, fungal hyphae were present in these lesions and were peroxidase positive before and after the periderm appeared and tannin accumulated in the cells. Necrotic areas occurred on seedlings of slash pine families regardless of the resistance in greenhouse or field tests.

In most developing galls on slash pine, necrotic areas merged with nonpigmented infected tissues (Fig. 1-B). The necrotic area formed between the epidermis and cambium near the inoculation site. A periderm did not form. Necrotic zones continued to increase as long as 60 days after inoculation (Fig. 2). Between 60 and 90 days the area of necrosis decreased while tissue affected by the

fungus increased rapidly. Necrosis often involved a needle trace. In necrotic areas, fungal hyphae ramified in and were continuous with hyphae in cambial cells after 30 days. Hyphae were positive for peroxidase in and around the necrotic area; host cells contained tannin and tannin-precursors.

Other extensive host cell necrosis occurred in the pith, xylem, and cortex (Fig. 1-C, 1-D, 1-E) of developing galls on slash pine. The necrosis was associated with normal growth of the fungus. The necrotic areas included as few as 10 and as many as several hundred cells in transverse sections. Hyphae were positive for peroxidase, and host cells contained tannin. Periderms were not present. Seedlings containing these necrotic areas had fungal hyphae in their cambial cells and in actively growing galls.

About 70% of the galled stems from older seedlings (9-14 mo) of loblolly, longleaf, and slash pines showed

TABLE 1. Incidence of necrotic areas, fungus hyphae, or both in galls on loblolly and slash pines 9 mo after inoculation<sup>a</sup>

Trait <sup>b</sup>	Incidence of disease traits by gall length ranges:					
	1-10 mm		11-29 mm		> 29 mm	
	L <sup>c</sup>	S <sup>d</sup>	L	S	L	S
Necrosis	78	100	91	90	84	93
Fungus in cambium	28	0	76	16	98	69
Fungus in border cells only	15	0	18	45	2	24
No fungus	57	100	6	39	0	7

<sup>a</sup>Galls on loblolly pine and galls on slash pine (150 each) were selected at random from materials embedded in paraffin.

<sup>b</sup>Specimens with fungus in the cambium had hyphae in other tissues as well. The "no fungus" category includes galls that contained fungal hyphae restricted to tannin-filled cells.

<sup>c</sup>L = loblolly pine.

<sup>d</sup>S = slash pine.

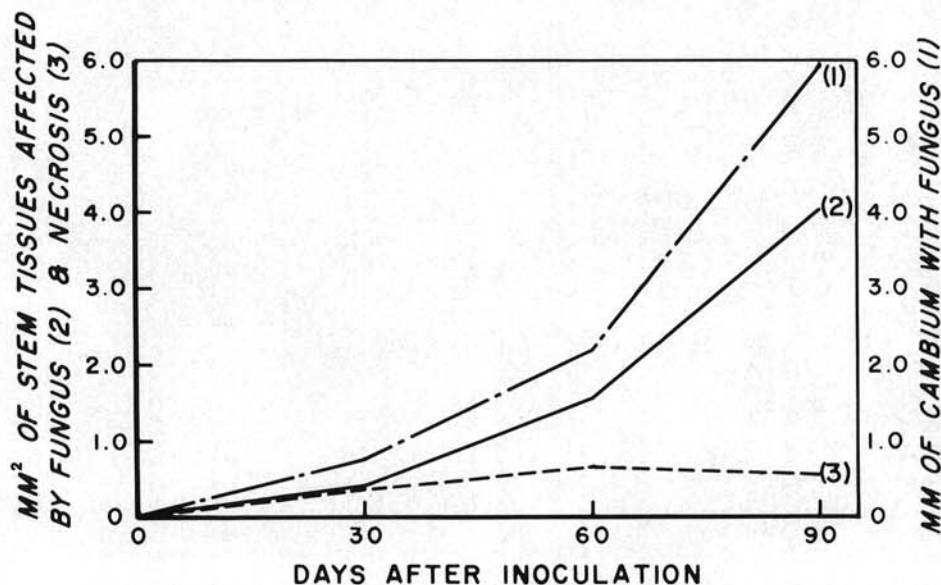
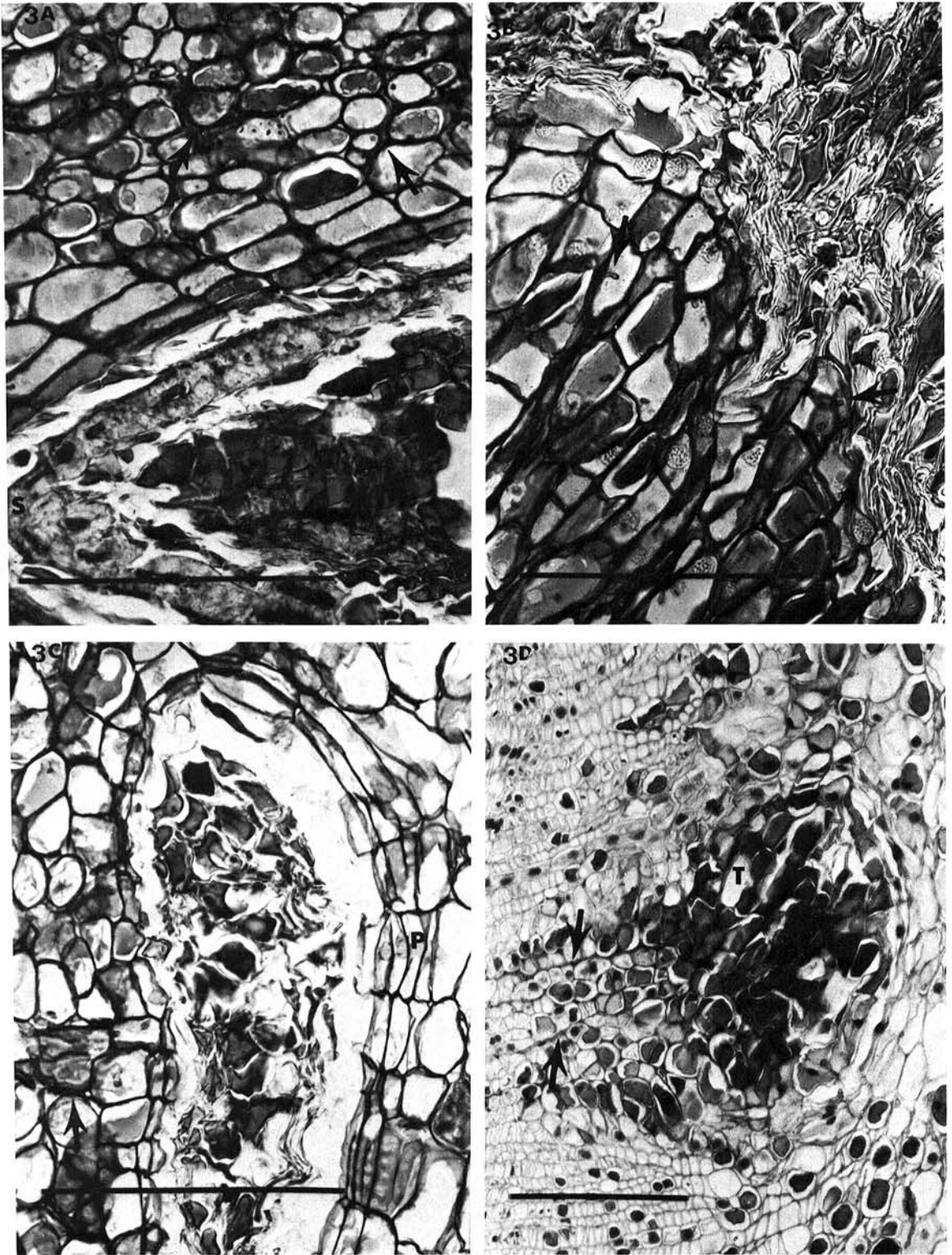


Fig. 2. Pine cell necrosis and growth of rust fungus in transverse sections of slash pine tissues. Values are averages of 10 galled seedlings for each time period. Note coincidence of mm<sup>2</sup> tissue area affected by fungus and cell necrosis at 0-30 days after inoculation. By 90 days the fungus encircled the cambium (6-mm circumference).



**Fig. 3.** Variations in the appearance of the border of necrotic areas in 7–12 mo slash and loblolly pine galls. **A)** Layer of stone cells (S). Fungal hyphae are evident (arrow). **B)** Aborted xylem elements with haustoria (arrow). **C)** Necrotic area bordered by a typical periderm (P). Hyphae (arrow) occur in large numbers on cambial side. **D)** Necrosis with tannin-filled cells (T) adjacent to healthy ones. Hyphae are in necrotic area (arrow). Scale bars represent 200  $\mu$ m.

tannin and necrosis. There was no significant difference in the incidence of necrotic areas in loblolly or slash pine families from Bent Creek or Gulfport.

Some necrosis occurred in nearly 300 loblolly and slash pine galls, regardless of their size (Table 1). Eighty-three percent of galls on longleaf pine seedlings that contained rust hyphae in the cambium exhibited necrotic areas.

Necrotic areas in infected 9 to 14 mo old seedlings occurred in all stem tissues but were most common in the cortex. Necrosis occasionally extended from the cortex to the inner xylem and pith. In loblolly pine and infrequently in slash and longleaf pines, portions of the pith were missing or appeared as a network of tannin-containing and distorted cell walls. Some galled stems were exposed from the outer bark to the pith because of destruction of a radial section of the tissues. The large amount of necrosis that occurred in longleaf pine did not hinder proliferation of the rust fungus. The shape and appearance of the tannin-containing areas in the cortex and xylem of older seedlings that had actively growing galls generally were similar to those reported by Jewell and Speirs (11) and Miller et al (16) for resistant seedlings.

Four types of borders were observed in galled tissues (Fig. 3). First, layers of sclereids formed the outside border of zones in the inner cortex and the inside border of zones that were being sloughed to the outside (Fig. 3-A). Second, many layers of distorted xylem cells that contained haustoria formed the inside border of necrotic zones that extended from the cortex to the xylem (Fig. 3-B). These cells often had a deeply-stained cytoplasm. Third, a periderm surrounded some necrotic zones in the cortex (Fig. 3-C). Fourth, cells containing tannin were adjacent to unpigmented cells that had no specialized border (Fig. 3-D).

More fungus occurred in loblolly pine galls that contained large necrotic zones (occupying 15-20% of the gall transverse area) than in slash pines (Table 1). As is evident from Table 1, small galls contained less fungus than the large ones. Actively growing galls that were free of necrotic zones contained abundant hyphae, as originally described by Jackson and Parker (10) for loblolly pine and Jewell et al (12) for slash pine.

#### DISCUSSION

Cell necrosis and tannin accumulation occur in a large percentage of actively growing galls formed from fusiform rust inoculations. The variety of necrotic areas in younger and older galled seedlings was similar to that described for resistant seedlings (11, 16). Such reactions occur in noninfected pine callus cultures (1, 7). The disruptions in cellular and tissue anatomy closely resemble that caused by wounding with fine pins (21).

With the possible exception of a necrotic area surrounded by a periderm (Fig. 1-A), the host response does not appear to be of the hypersensitive type (8). Positive peroxidase activity in the necrotic areas was accepted as evidence that the fungus was alive. The presence of abundant tannin near apparently living hyphae was described by Boyer (3) for white pine blister rust. Tannins are contained in membrane-bound organelles of plant cells and apparently do not diffuse outward (1, 5). This was evident in necrotic areas that had

healthy cells adjacent to tannin-filled ones.

The varied anatomy and size of necrotic areas in loblolly, longleaf, and slash pine galls suggest that at least two processes cause those areas. The first, described by True (22), Jewell and Speirs (11), and Miller et al (16), is a stimulation of meristematic cells, which gives rise to a variety of distorted cells. Eventually these can become necrotic. The second process is an alteration of existing parenchymal cells. This can occur in the cortex, pith, and rays, as described for white pine blister rust in tissue culture (17, 18). Necrosis and tannin formation in the pith cells are particularly interesting since they form a core of dead cells that are detected throughout the life of the tree.

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