

## Interactions Between Actinomycete-Like Organisms and Young Apple Roots Grown in Soil Conducive to Apple Replant Disease

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### ABSTRACT

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Prokaryotic mycelial organisms, similar to actinomycetes, were consistently found in lesions on young apple roots after seedlings had grown for 2 wk in soil conducive to apple replant disease. Although other organisms were found in the lesions, only the actinomycete-like hyphae were seen at the advancing lesion margins. These hyphae had penetrated walls of living cortical cells and were sometimes found associated with papillae at penetration sites in the cells. By 3 wk, invaded tissues had collapsed and actinomycete-like hyphae had penetrated to the endodermis. Further penetration was limited by the periderm, and infected tissues were sloughed as roots expanded in diameter. These observations indicate that

the actinomycete-like organisms actively invaded and damaged living cells of the root epidermis and cortex and that they should therefore be considered pathogenic. Rhizosphere soil collected from roots of seedlings grown in a soil conducive to apple replant disease promoted high levels of infection by the actinomycete-like organisms. Severity of infection of roots was associated with inhibition of both apple seedling growth and lateral root development, which are symptoms that have been associated also with apple replant disease. These results support other evidence indicating a causal role for organisms similar to actinomycetes in apple replant disease.

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The term apple replant disease (ARD) has been used to refer to many problems that result in delayed establishment of apple trees on sites that had been planted previously to apple. Symptoms include diminished growth, deterioration of young roots, and inhibition of lateral root development during the first few years after transplanting (6). These symptoms are not specific to ARD, so the causes must be determined for each situation. This is not always practical because all causes have not been identified. Several workers have demonstrated that some forms of ARD involve biotic agents (6,9,17), and the present investigation was limited to these. Thus, all references to the term "apple replant disease" refer to the field disease, described as "specific apple replant disease" by Hoestra (6), which has a biotic component in its etiology.

Otto and Winkler (14,15) presented evidence implicating actinomycete-like organisms as possible causal agents of ARD in East Germany. They stained roots from sites considered maximally, moderately, and minimally conducive to ARD and found that the extent of colonization of the outer tissues by actinomycete-like organisms was positively correlated with disease severity (14). Identification of the organisms in roots was based primarily on the small size of hyphae. Similar evidence was

obtained for fruit-tree nurseries in New York (25). Five nursery soils previously planted to apple or pear were determined to be conducive to ARD, and apple seedlings grown in the soils became infected by actinomycete-like organisms. Conversely, soils not previously planted to apple in the known history of a site were not conducive to ARD, and seedlings planted in the soils did not become infected. Additionally, Otto and Winkler (15) found that certain pesticides that reduced the number of propagules of actinomycetes recoverable from soil were also effective in controlling ARD. However, they did not attempt to isolate the actinomycetes that actually invaded the apple roots (*personal communication*, 1984).

Because it appeared that actinomycetes might be involved in the etiology of ARD in New York (23,25), we examined infected roots microscopically to characterize the filamentous organisms in apple roots from seedlings grown in soil conducive to ARD and to determine the nature of the interaction between these organisms and apple roots. Soil infestation trials also were conducted to relate root infection by the actinomycete-like organisms with inhibition of growth associated with ARD.

### MATERIALS AND METHODS

**Soil.** Soil was collected from around apple tree roots in a commercial orchard in Wayne County, NY, at a site shown to be

conductive to apple replant disease (9,13). This soil (Smith soil), which was stored at 4 C until used, was a loamy sand (pH 7.1) from the Alton gravelly sandy loam series (7). Samples of the same soil were treated with aerated steam at 60–70 C for 30 min (steamed soil) and used as the base soil in experiments.

**Preparation of root tissue for microscopy.** Apple seedlings were grown in Smith soil or steamed soil for 2 to 3 wk in a controlled environment chamber (21 C, 14 hr light, 40–75% RH). Roots were then harvested and immersed in water so that adhering soil could be removed with a fine sable-hair brush. Pieces of roots (about 1 cm long) were selected from seedlings and fixed for 1 hr in 5% (v/v) glutaraldehyde in 0.07 M potassium phosphate buffer, pH 6.8, at 21 C. The tissue was rinsed four times in the same buffer and postfixed in 2% (w/v) osmium tetroxide in buffer for 1 hr. After three rinses in buffer and three in water, the tissues were dehydrated via a graded acetone series and then rinsed in propylene oxide six times over a 2-hr period. Infiltration with Spurr's low viscosity resin mix E (18) was achieved over 2 days using a graded resin series prepared in propylene oxide and cured for 16 hr at 70 C. Thick sections, 20–40  $\mu$ m, from three roots were cut and then examined with differential interference contrast optics (2). Selected thick sections then were remounted on plastic blocks and thin-sectioned at 60 nm. Sections were stained for 5 sec with 2% (w/v) uranyl acetate in methanol and sections from two separate lesions were examined with a Philips EM 200 transmission electron microscope.

**Seedling bioassay methods.** Seeds that had been collected from Northern Spy apples were stratified under moist conditions at 4 C for 3 mo. Germinated seeds, selected for uniform length and diameter of the emerging radicle, were planted in steamed soil or various infested soils in seedling tray cones and grown in a controlled environment chamber (21 C, 40–75% RH, 14 hr light) for 2 wk. The individual cones were 2.5 cm<sup>2</sup> at the top and tapered to 0.5 cm<sup>2</sup> at a depth of 8 cm. Trays were placed on flats of moist vermiculite. At harvest, seedlings were washed from each cone with a gentle stream of water. Seedlings without a taproot and those exhibiting symptoms of the lethal gene syndrome (22) were discarded at harvest. Rinsed and blotted seedlings were weighed, and the numbers of lateral roots were counted.

Roots were stained to reveal infection by actinomycete-like organisms in some experiments. The staining procedure used was modified from that reported by Kormanik et al (12) for visualization of endomycorrhizal fungi in roots. Roots were cleared in 1 N KOH in an autoclave at 121 C for 3 min and then were rinsed in water until the rinse water remained colorless. Roots were then treated for 3 min in 1 N HCl, drained and immersed in 0.05% (w/v) trypan blue in a 100:7:7 lactic acid-glycerol-water solution (v/v/v) for 8–15 hr at room temperature. After staining, roots were stored in glycerol. Actinomycete-like hyphae stained dark blue in the epidermis and cortex of roots. Confirmation of actinomycete infection was made by examining samples for fine hyphae (about 1  $\mu$ m diameter) at 1,000 $\times$ . The percent root epidermal area colonized by the actinomycete-like organism was estimated on 1-cm increments of the taproot at 15 $\times$  magnification by reference to standard diagrams (Key No. 2.1.1) (10).

**Infestation trials.** Apple seedlings were grown in either steamed soil, Smith soil, or a mixture of steamed soil with Smith soil at the rate of 4:1 (v/v), respectively. For the soil mixture, water was added to Smith soil (50 ml/100 cm<sup>3</sup>) before it was mixed with about 400 cm<sup>3</sup> of dry, steamed soil. The other soils were moistened with water (50 ml/500 cm<sup>3</sup>) and mixed thoroughly. Three replicates of each treatment were planted with 18 apple seeds for a seedling bioassay experiment.

Rhizosphere soil was collected from 500 apple seedling roots that had grown in Smith soil for 19 days. First, large clumps of soil were shaken gently from roots and discarded. Next, the roots were rinsed in water for 30 min with gentle agitation to dislodge the rhizosphere soil, which was then allowed to settle for 1 hr before excess water was decanted. Lastly, collected soil was air dried until a workable moisture level was obtained (about 10%, w/w).

Rhizosphere soil (54-, 5.4-, and 0.5-g portions on a dry weight basis) was mixed separately in 50 ml of water. One set of each

rhizosphere soil suspension was held at 60 C for 30 min. The suspensions then were each mixed with steamed soil to make up 600 cm<sup>3</sup> of soil and distributed among 20 cones in seedling trays for each treatment. A 2-wk seedling bioassay was conducted as described above.

## RESULTS

**Microscopy.** Under the conditions of our experiments, apple seedling roots planted in soil conducive to ARD were consistently infected by filamentous organisms; those planted in steamed soil were not. Light brown superficial lesions developed on infected roots, and microscopic examination of lesions consistently revealed hyphae about 1  $\mu$ m diameter. The light micrographs shown in Figures 1–4 illustrate the appearance of lesions and the actinomycete-like hyphae in the epidermis and cortex of apple seedling roots. The advancing edges of lesions were consistently semicircular in cross section (Fig. 1). Epidermal and cortical cells in the lesion were filled with hyphae (Figs. 3 and 4). Only hyphae similar in size to those of an actinomycete were observed in the invaded cortical cells at the margins of lesions. In five lesions, cortical cell nuclei often were located on the wall proximal to the adjacent invaded cell (Fig. 3). In some sections, rod-shaped bacterial cells and larger hyphae, which were presumed to be fungal, were present on the surface of roots (Fig. 4).

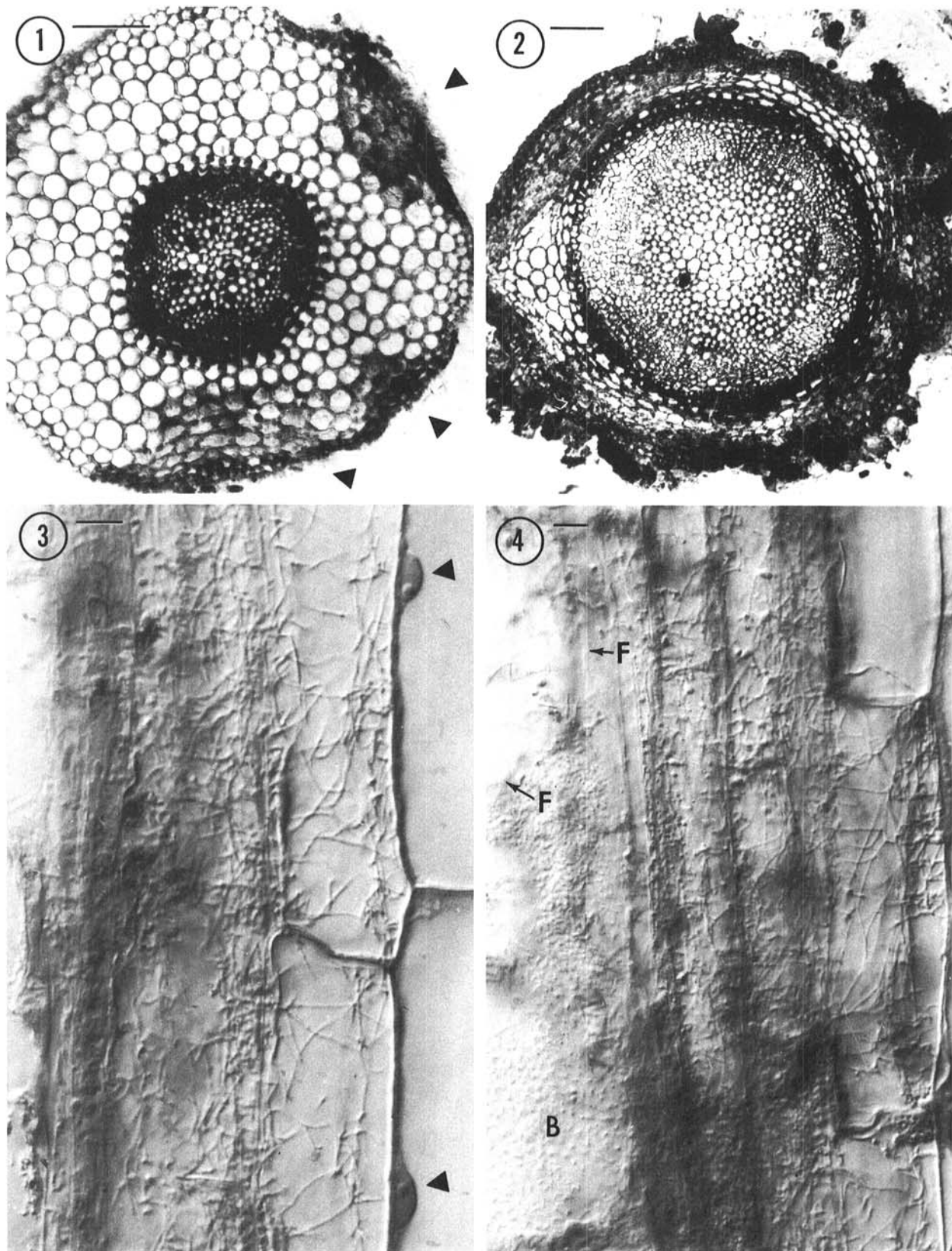
In tissues of 3-wk-old roots, only epidermal and cortical tissues that had been invaded by the actinomycete-like organisms had collapsed (Fig. 2). There was no evidence of penetration of the periderm, and direct examination of roots indicated that the infected tissues were sloughed as roots expanded during secondary growth. For roots grown in steamed soil for 3 wk, the epidermis and cortex were split due to growth of the periderm, but the cortical tissues had not collapsed.

Electron micrographs of root tissues near the margin of a lesion revealed hyphae approximately 1  $\mu$ m in diameter and without organelles (Fig. 5). The hyphae were branched and contained septa. Hyphal penetration of root cell walls was observed frequently. Penetration of cortical cell walls at the margins of lesions was observed. These cortical cells contained intact cell organelles. The hyphae contained mesosomes that were associated frequently with the tips of hyphae where they had penetrated plant cell walls. Papillae often were seen around hyphae (Fig. 6) but not always where penetration of cell walls had occurred (Fig. 7). Papillae also had formed at sites remote from any hypha that had penetrated a cell wall but always in cortical cells near the margin of the lesions (Fig. 8). In older parts of the lesion, where hyphae were numerous, parts of host cell walls were missing.

**Infestation trials.** When Smith soil was added to steamed soil or used alone as the planting medium, seedling growth was reduced, compared with growth in steamed soil alone, and roots were infected by actinomycete-like organisms (Tables 1 and 2). The severity of infection by actinomycete-like organisms appeared to be roughly proportional to the quantity of Smith soil added. Small quantities of the rhizosphere soil caused significant inhibition of plant growth and associated infection by the actinomycete-like organisms (Table 3). Higher concentrations of rhizosphere soil resulted in more severe inhibition of seedling growth and infection by the actinomycete-like organisms.

## DISCUSSION

Actinomycete-like organisms were associated with apple seedling roots that had been grown in soil collected from an apple orchard. The organisms were observed in whole-root mounts stained with trypan blue. Ultrastructural features of the organisms indicated that they were mycelial procaryotes, similar to other actinomycetes (3,5,19,21). No distinctive morphological or ultrastructural features were observed that permitted identification of the species involved. Severe infection of seedling roots resulted from mixtures of soil containing small quantities of rhizosphere soil, which suggests that the actinomycete-like organisms may produce infectious propagules around infected roots. Microscopic



**Figs. 1-4.** Light micrographs of young apple roots grown in soil conducive to apple replant disease. **1,** A cross section through a whole root showing semicircular lesions (arrows) that had developed after 2 wk (scale bar = 100  $\mu$ m). **2,** After 3 wk, epidermal and cortical cells had collapsed around most of the circumference of the root. Note that there was little penetration of the endodermis until this stage and the developing periderm was not penetrated (scale bar = 100  $\mu$ m). **3,** This radial section illustrates hyphal ramification in cells and two instances where cortical cell nuclei (arrows) were addressed to the wall proximal to the invaded cells (scale bar = 10  $\mu$ m). **4,** On the left are rod-shaped bacterial cells from a colony (**B**) that was on the surface of the root before sectioning. Hyphae, similar to those of fungi (**F**), are in the upper left corner. Only hyphae similar in size to those of actinomycetes were visible in the innermost infected cells at the right of the illustration (scale bar = 10  $\mu$ m).

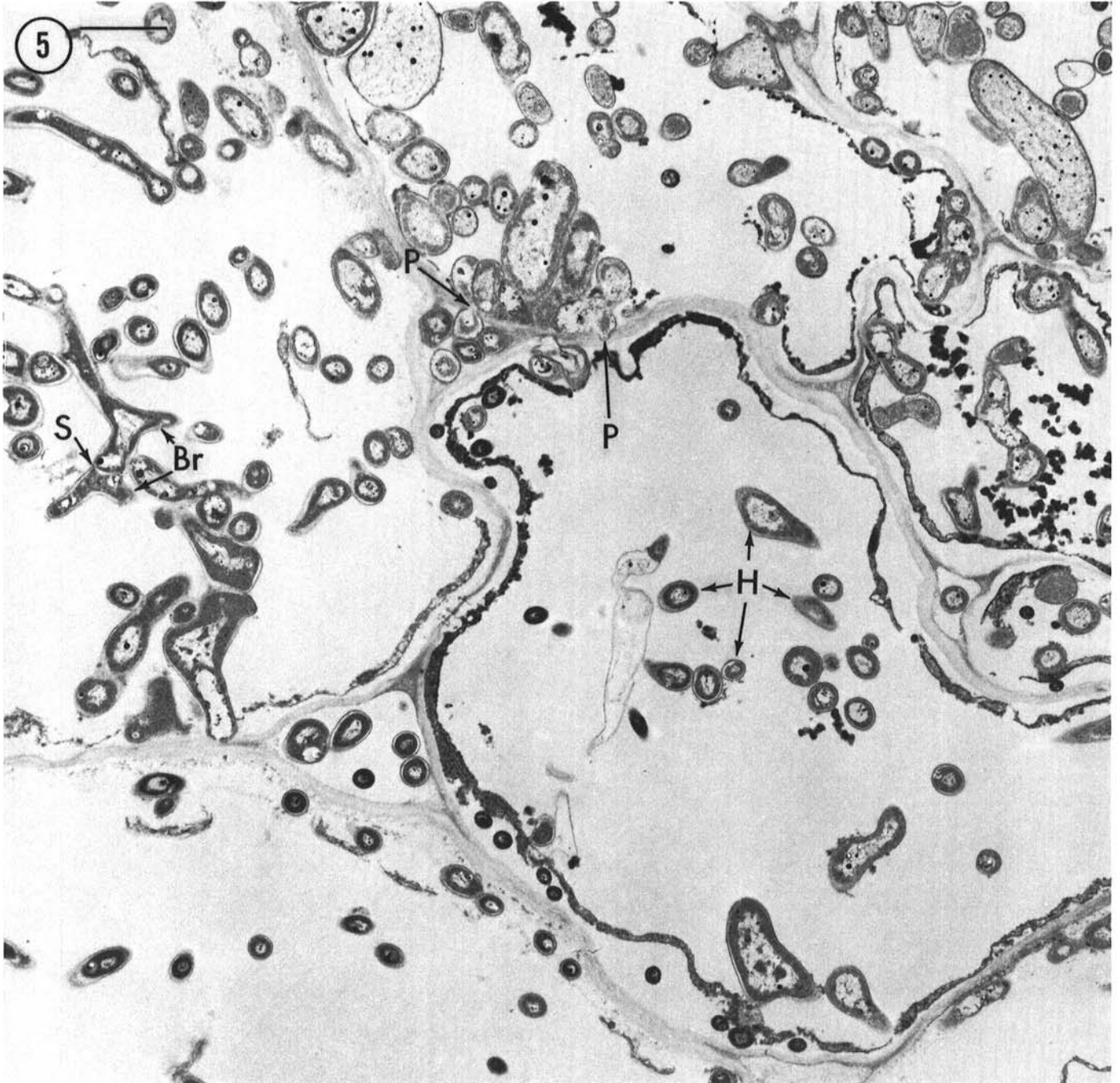
identification of spores associated with the actinomycete-like organisms was not possible due to presence of many saprophytes on the surface of infected roots.

The studies reported here were carried out with Smith soil that had been collected from one site conducive to ARD. In another study (25), observations of trypan-blue-stained apple seedling roots that had been grown in soils collected from several additional sites indicated the presence of similar symptoms and signs as seen in detail in roots grown in Smith soil. Those results suggest that the association of actinomycete-like organisms is not restricted to apple seedling roots grown in one particular soil but may be a widespread phenomenon.

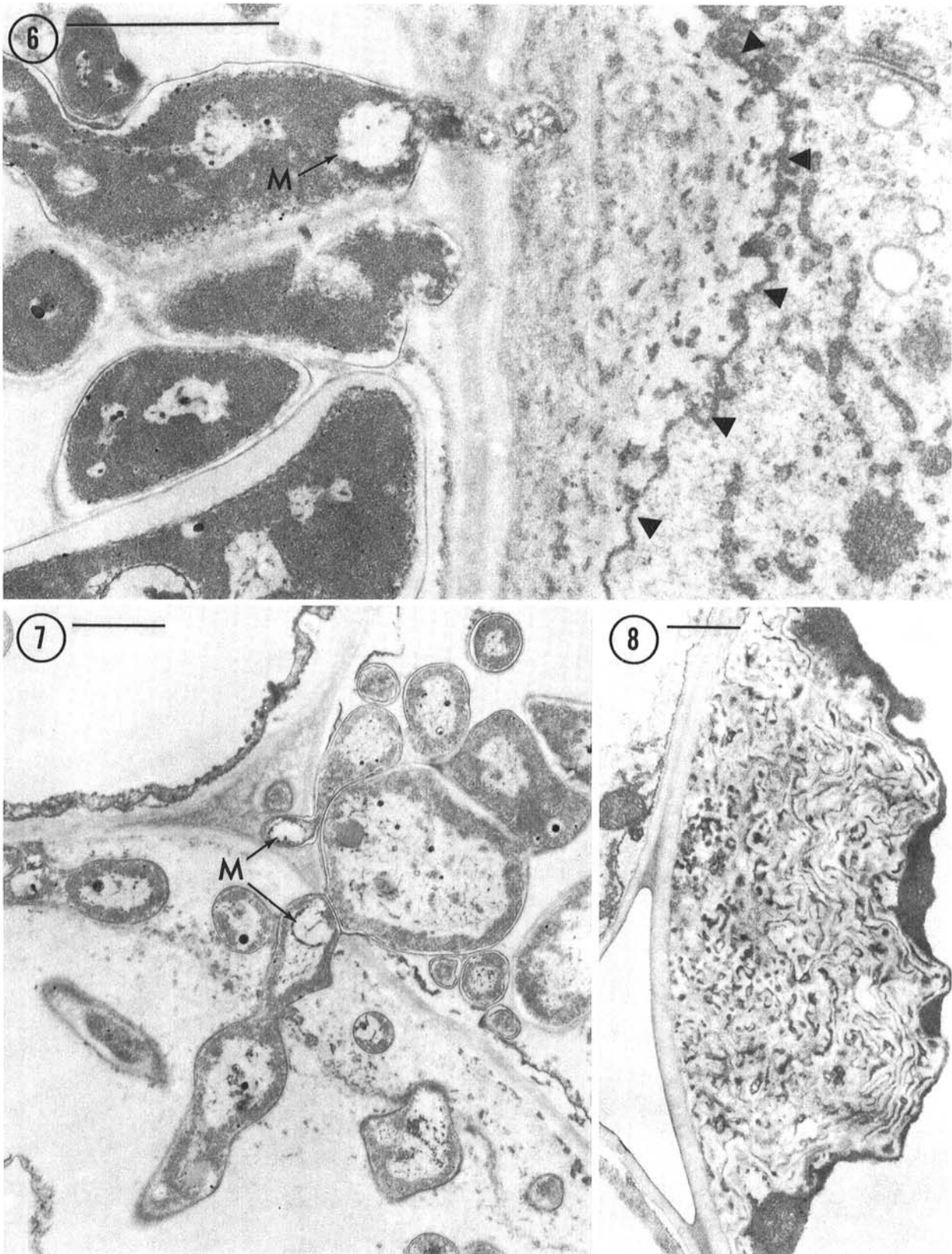
The evidence suggests strongly that the actinomycete-like organisms are pathogenic. Light brown superficial lesions were consistently associated with sites of invasion of roots by actinomycete-like organisms. Actinomycete-like hyphae were

found, alone, in the cortical cells at the margins of lesions. Hyphae that had penetrated walls of cortical cells with intact cell organelles were observed frequently. The ultrastructure of these cortical cells was similar to cells from healthy root tissues. Thus it appeared that the actinomycete-like organisms were able to invade intact root tissues. In general, actinomycetes grow too slowly to compete with fungi and bacteria for simple carbohydrates and are involved typically in breakdown of complex compounds such as cellulose, hemicellulose, and lignin (19,21). If another organism(s) injured plant cells in advance of invasion of tissues, actinomycetes would not be expected to be the sole organisms observed at the margins of lesions. On the contrary, the mere existence of actinomycete-like organisms in the root cortex ahead of other organisms is strong evidence that they are pathogenic.

Cytological evidence also supports the pathogenic association between actinomycete-like organisms and young apple roots.



**Fig. 5.** An electron micrograph of cortical cells in a lesion on an apple root grown for 2 wk in soil conducive to apple replant disease. Hyphae (H) occurred in cortical cells and in intercellular spaces. Cytoplasm in these infected cells was partially disintegrated. Hyphae were branched (Br) and septate (S). Two points of penetration (P) of the cortical cell walls are indicated (scale bar = 2  $\mu$ m).



**Figs. 6-8.** Electron micrographs of hyphae and plant cell structures in cortical cells (all scale bars = 1  $\mu$ m). **6,** Hyphae have penetrated a cell wall of a cortical cell at the margin of the lesion, and a papilla formed around the site of hyphal penetration (arrows). Note that plant cell organelles are intact in the uninvaded cortical cell to the right. A mesosome (M) is present in the tip of the hypha in close proximity to the papilla. **7,** A penetration site in a cortical cell wall where no papilla formed. A second hypha penetrated the intercellular space next to the first hypha. Mesosomes (M) were visible in both. **8,** A papilla, which was not closely associated with any actinomycete-like hyphae, formed in a cortical cell next to an infected cell to the left.

TABLE 1. Plant growth and infection of roots by actinomycete-like organisms of apple seedlings grown in a soil conducive to apple replant disease (Smith soil) mixed with steamed Smith soil (20%, v/v) or steamed Smith soil alone<sup>a</sup>

| Trial no.                     | Soils                | Plant weight (mg) | Lateral roots (no.) | Actinomycete infection <sup>b</sup> (%) |
|-------------------------------|----------------------|-------------------|---------------------|---|
| 1                             | Mixture <sup>c</sup> | 285 ± 26          | 45 ± 7              | 8 ± 3                                   |
|                               | Steamed soil         | 407 ± 54**        | 54 ± 6*             | 0                                       |
| 2                             | Mixture <sup>c</sup> | 326 ± 32          | 42 ± 6              | 5 ± 2                                   |
|                               | Steamed soil         | 465 ± 38**        | 58 ± 6**            | 0                                       |
| 3                             | Mixture <sup>c</sup> | 327 ± 33          | 40 ± 6              | 14 ± 3                                  |
|                               | Steamed soil         | 410 ± 28**        | 50 ± 4*             | 0                                       |
| Protected LSD <sub>0.05</sub> |                      | 44                | 8                   | ...                                     |
| Protected LSD <sub>0.01</sub> |                      | 58                | 11                  | ...                                     |

<sup>a</sup> Means and 95% confidence limits are given for each parameter in the table. Significant differences between unsteamed and steamed treatments are indicated: \* at the 0.05 level; \*\* at the 0.01 level.

<sup>b</sup> The values are the percent root epidermal area colonized by actinomycete-like organisms along the top 6 cm of the taproot. Means were not compared because there was no infection of roots grown in steamed soil treatments.

<sup>c</sup> A mixture of 20% (v/v) Smith soil with steamed Smith soil.

TABLE 2. Plant growth and infection of roots by actinomycete-like organisms of apple seedlings grown in a soil conducive to apple replant disease (Smith soil) or steamed Smith soil<sup>a</sup>

| Trial no.                     | Steam treatment of soils | Plant weight (mg) | Lateral roots (no.) | Actinomycete infection <sup>b</sup> (%) |
|-------------------------------|--------------------------|-------------------|---------------------|---|
| 1                             | no                       | 249 ± 23          | 16 ± 4              | 41 ± 13                                 |
|                               | yes                      | 342 ± 27**        | 37 ± 5**            | 0                                       |
| 2                             | no                       | 259 ± 20          | 20 ± 3              | 52 ± 13                                 |
|                               | yes                      | 369 ± 21**        | 38 ± 3**            | 0                                       |
| 3                             | no                       | 244 ± 24          | 17 ± 4              | 30 ± 5                                  |
|                               | yes                      | 333 ± 31**        | 32 ± 4**            | 0                                       |
| Protected LSD <sub>0.01</sub> |                          | 41                | 7                   | ...                                     |

<sup>a</sup> Means and 95% confidence limits are given for each parameter in the table. Significant differences between unsteamed and steamed treatments at the 0.01 level are indicated with \*\*.

<sup>b</sup> The values are the percent root epidermal area colonized by actinomycete along the top 6 cm of the taproot. Means were not compared because there was no infection of roots grown in steamed soil treatments.

TABLE 3. Plant growth and infection of roots by actinomycete-like organisms of apple seedlings grown in steamed soil mixed with untreated or heated rhizosphere soil conducive to apple replant disease<sup>a</sup>

| Weight (g) of rhizosphere soil added per 600 cm <sup>3b</sup> | Heat treatment of soils <sup>c</sup> | Plant weight (mg) | Lateral roots <sup>d</sup> (no.) | Actinomycete infection <sup>e</sup> (%) |
|---|--------------------------------------|-------------------|----------------------------------|---|
| 0.5   | no                                   | 290               | 31                               | 13                                      |
|   | yes                                  | 320               | 38*                              | 0                                       |
| 5.4   | no                                   | 280               | 24                               | 52                                      |
|   | yes                                  | 340**             | 40**                             | 0                                       |
| 54.0  | no                                   | 230               | 18                               | 73                                      |
|   | yes                                  | 340**             | 39**                             | 0                                       |
| Protected LSD <sub>0.05</sub>                                 |                                      | 40                | 7                                | ...                                     |
| Protected LSD <sub>0.01</sub>                                 |                                      | 50                | 9                                | ...                                     |

<sup>a</sup> Significant differences between means for unheated and heated soils are indicated: \* at the 0.05 level; \*\* at the 0.01 level.

<sup>b</sup> Rhizosphere soil was collected from 500 seedlings that had been grown for 19 days in Smith soil.

<sup>c</sup> Rhizosphere soil in 50 ml of water was heated to 60 C for 30 min.

<sup>d</sup> The number of lateral roots on the taproot was counted from 1 to 5 cm below the root crown of each seedling.

<sup>e</sup> Estimates of the root epidermal area of the taproot colonized by the actinomycetes from 1 to 5 cm below the root crown are expressed as percentages. The variances were not uniform among the treatments, so analysis of variance was not performed; standard deviations were 22, 46, and 58 for treatments receiving 0.5, 5.4, and 54 g of untreated rhizosphere soil, respectively.

Nuclei in cortical cells were oriented toward nearby infected cells. Such nuclear reorientations have been described as typical responses of injured and infected tissues in other diseases (4, 16). Penetrations of epidermal and cortical cell walls indicate that the actinomycetes have the necessary mechanisms for penetration of plant cell walls and are not inhibited by possible plant defense mechanisms. On several occasions, papillae encased tips of actinomycete-like hyphae; presumably they formed in response to penetration of a root cell by the hyphae. Papillae, which were not associated with hyphae, also were observed in cells near infected cells. These observations are important because they provide evidence of an active response by the plant to invasion by the actinomycete-like organisms, as has been described for many fungal plant pathogens (1, 20). The presence of papillae separate from hyphae may even indicate release of injurious products by the microorganism (1).

Infested soil contained many microorganisms in addition to actinomycetes, so it was impossible to determine whether the actinomycete-like organisms could infect roots in the absence of these other organisms. Although the actinomycete-like organisms appeared, primarily on histological evidence, to be pathogenic, the extent of plant injury by them was not determined in a monoxenic system.

An argument against the observed actinomycete-like organisms as the cause of ARD might include the observation that invasion of root tissues was limited to the epidermis and cortex. Although premature destruction of these tissues could affect plant growth, the damage should not cause the severe growth inhibition observed. Soil actinomycetes, for example, species of *Streptomyces* and *Nocardia* (11), have been shown to produce substances having plant hormonal activity. Thus, the actinomycete-like organisms infecting apple roots also might produce a substance that affects plant growth. The cytological evidence (above) supports a hypothesis involving release of substances injurious to plant cells. Therefore, until counter evidence is available, actinomycetes cannot be dismissed as possible causal agents of ARD.

Infection of apple roots by actinomycete-like organisms occurred in this and other soils conducive to ARD (25). Our experiments have confirmed Jaffee's results that indicated the rhizosphere soil from affected roots promotes severe symptoms of ARD (8). We have shown also that severe infection of apple roots by actinomycete-like organisms occurs in soils mixed with rhizosphere soil. Fresh soils become conducive to ARD after rather short exposure to growing apple trees (6). This could be related to the physical distribution of rhizosphere soil.

The severity of infection of apple roots by actinomycete-like organisms appeared to be related to the degree of plant growth inhibition and lateral root development. This relationship implies that infection by the actinomycete-like organisms is related to inhibition of apple seedling growth and therefore is involved in the cause of this disease. This has been proposed and supported by Otto and Winkler (14, 15). However, definitive evidence is lacking. Proof of this hypothesis will require isolation, axenic cultivation of the actinomycete-like organisms, and controlled infestation of soils that are not otherwise conducive to ARD. Our first attempts to isolate actinomycetes have resulted in the isolation of only saprophytic actinomycetes (24).

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