Electron Microscopy in situ of the Bacterium
Associated with Ratoon Stunting Disease in Sundangrass

J. F. Worley and A. G. Gillaspie, Jr.

ABSTRACT

Basal stem tissue of sorghum-sudangrass hybrid uprights (two-node cuttings with the lower bud removed) with symptoms of ratoon stunting disease were sectioned for electron microscopy. These thin-sections revealed the presence of many rod-shaped bodies in the large xylem vessels. The bodies measure about 0.3 - 0.4 by 5-10 μm, and have a smooth cell wall indicative of a bacterium. The frequency of septa, and lack of evidence for binary fission, suggest that the organism belongs to the coryneform group of bacteria. A similar organism was seen, but far less often, in ratoon stunt-infected sugarcane.

Phytopathology 65:287-295

Additional key words: sugarcane, coryneform bacterium.

Under normal greenhouse and field conditions, ratoon stunting disease (RSD) causes few, if any, external symptoms on susceptible cultivars of sugarcane. Internal diagnostic signs occur in some cultivars, but their relationship to the disease agent is unknown. Recently, a bacterium or rickettsia-like organism was associated with diseased sugarcane (3, 8, 10). This organism was in xylem exudates, in juice expressed from canes, and in concentrated fibrovascular fluids (3, 10). In situ studies, however, have been very difficult. Maramorosch et al. (8) showed the presence of a rickettsia-like organism or a small bacterium in xylem vessels, but identification of the organism based on their micrographs was equivocal. We have had limited success in many attempts to see a possible disease agent in various parts of several sugarcane varieties. The apparent paucity of an organism in sugarcane led us to search for the RSD agent in another host that shows reliable symptoms.

In uprights of the sorghum-sundangrass hybrid NB 280S, symptoms of RSD are acute and are characterized by wilting and death (1, 2). As discussed by Benda (1) an upright is a two-node cutting planted vertically, with the basal bud removed so that the buried node will produce roots only, and the exposed one will produce the shoot. The shoot is supported on, and supplied by, the stalk and roots of the cutting. The symptoms on uprights are thought to be associated with the small diameter of the stem in the region where the shoot is attached to the cutting. Extracts from this region of diseased, wilted uprights contained large numbers of a bacteriumlike organism that is diagnostic for RSD (3). Thin-sections of this region from such uprights revealed large numbers of a bacterium to be present in xylem cells.

MATERIALS AND METHODS.—Uprights of RSD-diseased and healthy sudangrass hybrid NB 280S plants were grown in a greenhouse at the U.S. Sugarcane Laboratory, Houma, Louisiana. When the diseased uprights were wilting severely, they and the controls were sent to Beltsville for processing. Also, greenhouse and field-grown sugarcane, RSD-diseased and healthy control, of the cultivars CP 45-184, CP 53-1, Co 421, and Q28 (all interspecific hybrids of *Saccharum*), and Coimbatore (*S. spontaneum* L.) were grown in Houma and sent to Beltsville. Sugarcane cultivar CP 44-101 was grown in a greenhouse at Beltsville and was collected at various ages.

Samples of leaves, buds, stalk internodes, or nodes were fixed in 3% glutaraldehyde in 0.15 M phosphate buffer (pH 7) for about 4 hours at room temperature, rinsed briefly in buffer, and postfixed for about 2 hours in

2% osmium tetroxide in the same buffer at room temperature. After several rinses in buffer, the pieces were dehydrated in an ethanol:propylene oxide series and embedded in araldite resin. Sections were stained with uranyl acetate and lead citrate. Comparable material for light microscopy was fixed in formalin:acetic acid:ethanol (5:5:90, v/v), dehydrated in an ethanol:tertiary butanol series, embedded in Paraplast Plus, and stained with safranin and fast green.

RESULTS.—The six sugarcane cultivars were poor sources of material for finding a possible pathogen in situ by electron microscopy. Only one sugarcane plant thus far has yielded sections in which bodies are clear enough and numerous enough to indicate that they might be associated with the disease. This naturally infected, field-grown plant was of cultivar Co 421. Samples were taken from a lower node in an area where many vascular bundles were discolored. Bacteria or bacteriumlike organisms were seen in several of the large xylem vessels. The organism had a diameter of about 0.3 μm, a definite cell wall, and a membrane surrounding an electron-dense cytoplasm. The bodies were usually adjacent to a xylem cell wall, and most were in the partly confined space formed by the secondary heavy thickenings of the cell wall (Fig. 1). Although the bodies were bacteria or bacteriumlike, they were too few in number to allow identification or characterization.

On the other hand, sections of the RSD-diseased sudangrass hybrid had vessels that contained easily identifiable bacterial cells. It was not unusual to see five or six contiguous vessles nearly filled with bodies (Fig. 2). The bacteria were long rods, 0.3 - 0.4 by 5-10 μm, with clearly defined membranes and thin cell walls. Most of the walls were smooth, and only occasionally did they appear rippled. The smooth walls were most obvious on the longer segments, where the bodies were sectioned in nearly longitudinal planes (Fig. 3). The bacterial cytoplasms were not homogeneous. Many central areas were more electron-transparent and fibrillar than were the outer cytoplasms (Fig. 3 and 4). Occasionally the bacterium was branched. Often cells were separated by septa; sometimes, multiple cells formed a chain dissected by two or more septa (Fig. 3). We have seen no evidence of binary fission, as would be expected if the organism were a eubacterium. In many sections (such as that illustrated by Fig. 7) there were multiple cases of septa (coryneform bacterium), but no binary fission (eubacteria). Bacterial cells that were free of surrounding matrix showed no structure external to the walls (Figs. 3 and 7); however, when enclosed within a matrix, there appeared to be an extra outer layer (Fig. 4). In Fig. 4 beginning with the innermost light layer we interpret the alternate light and dark layers to be 1) space between the membrane and cytoplasm, 2) membrane, 3) space between the membrane and cell wall, 4) cell wall, and 5) space between the cell wall and the homogeneous matrix within the xylem
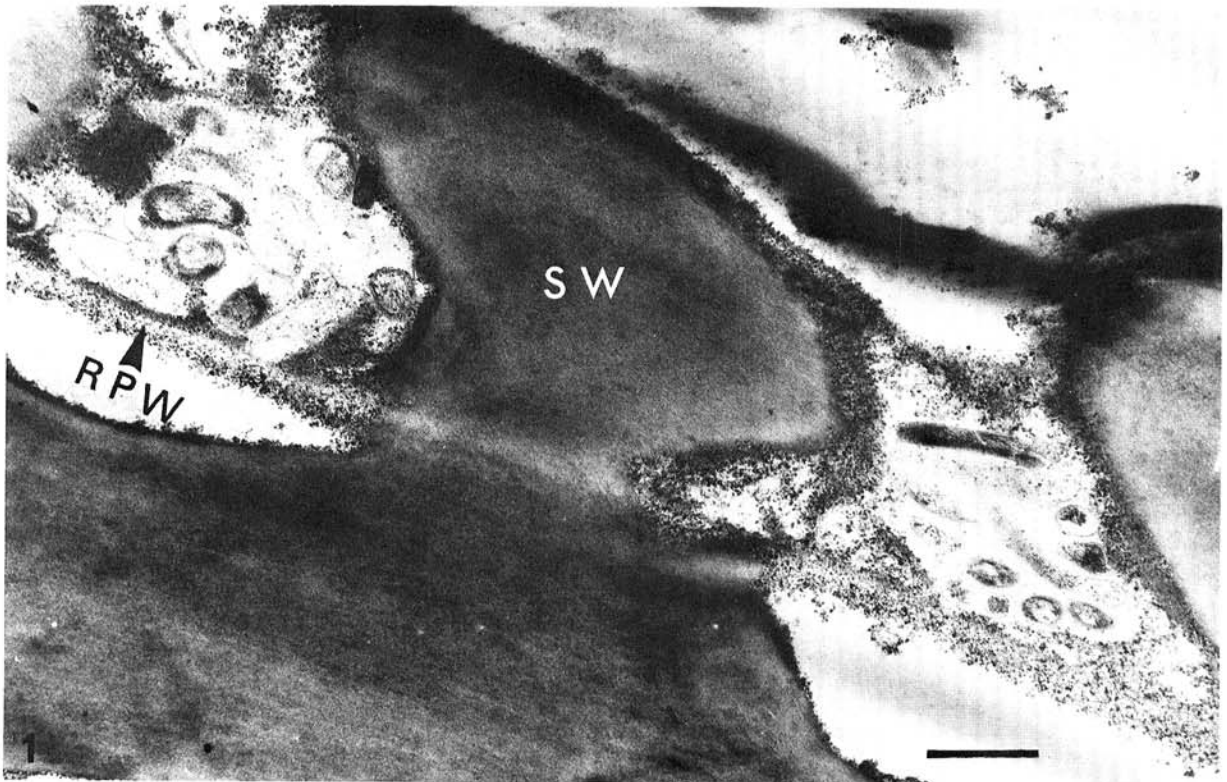

Fig. 1. Microorganism in xylem cells of ratoon stunting diseased sugarcane, cultivar Co. 421. SW = secondary wall. RPW = residual primary cell wall. Bar is approximately 1 μm.
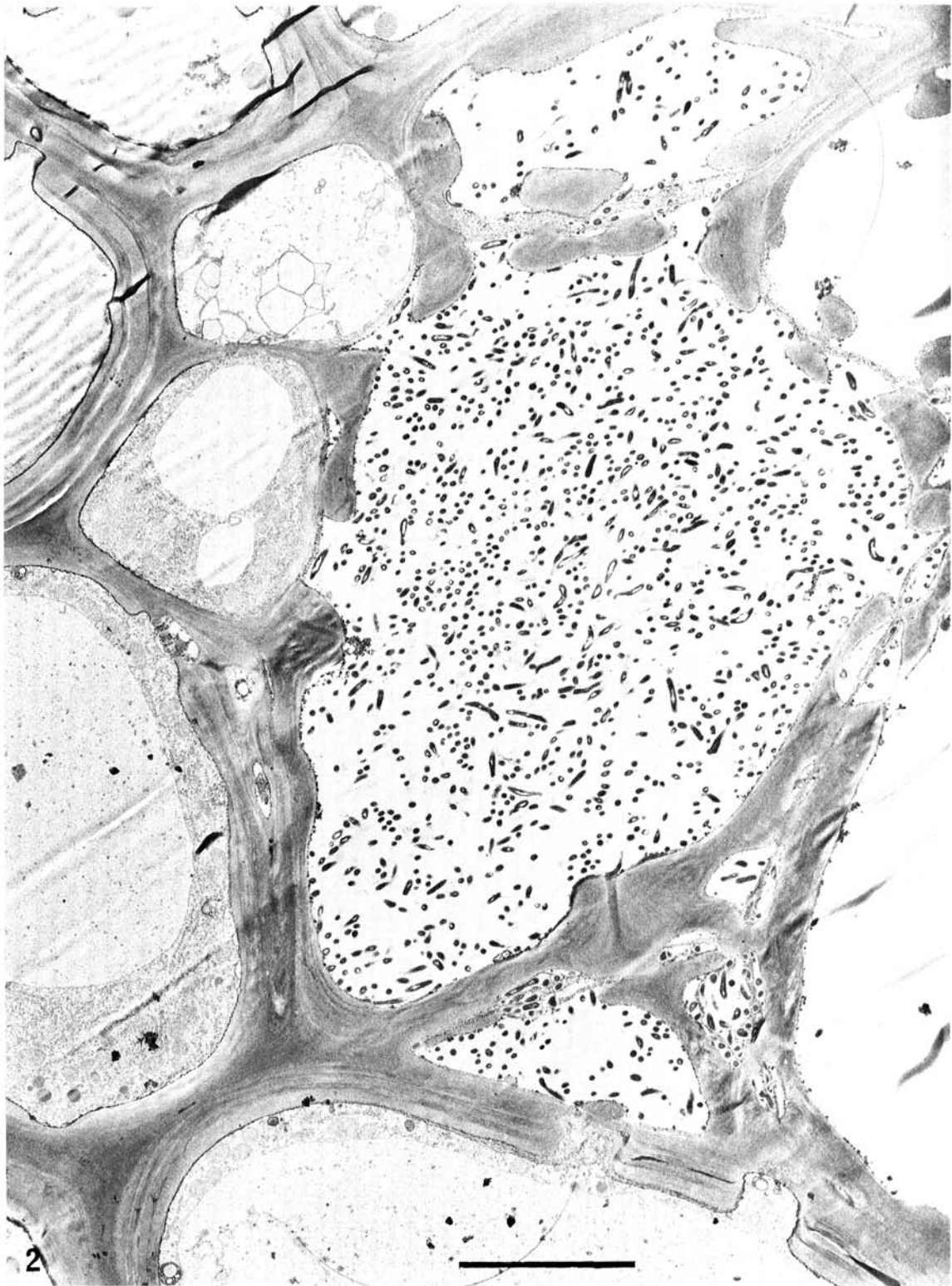
Fig. 2. Bacteria in contiguous xylem cells of ratoon stunting diseased sudangrass hybrid. Bar is approximately 100 μm.
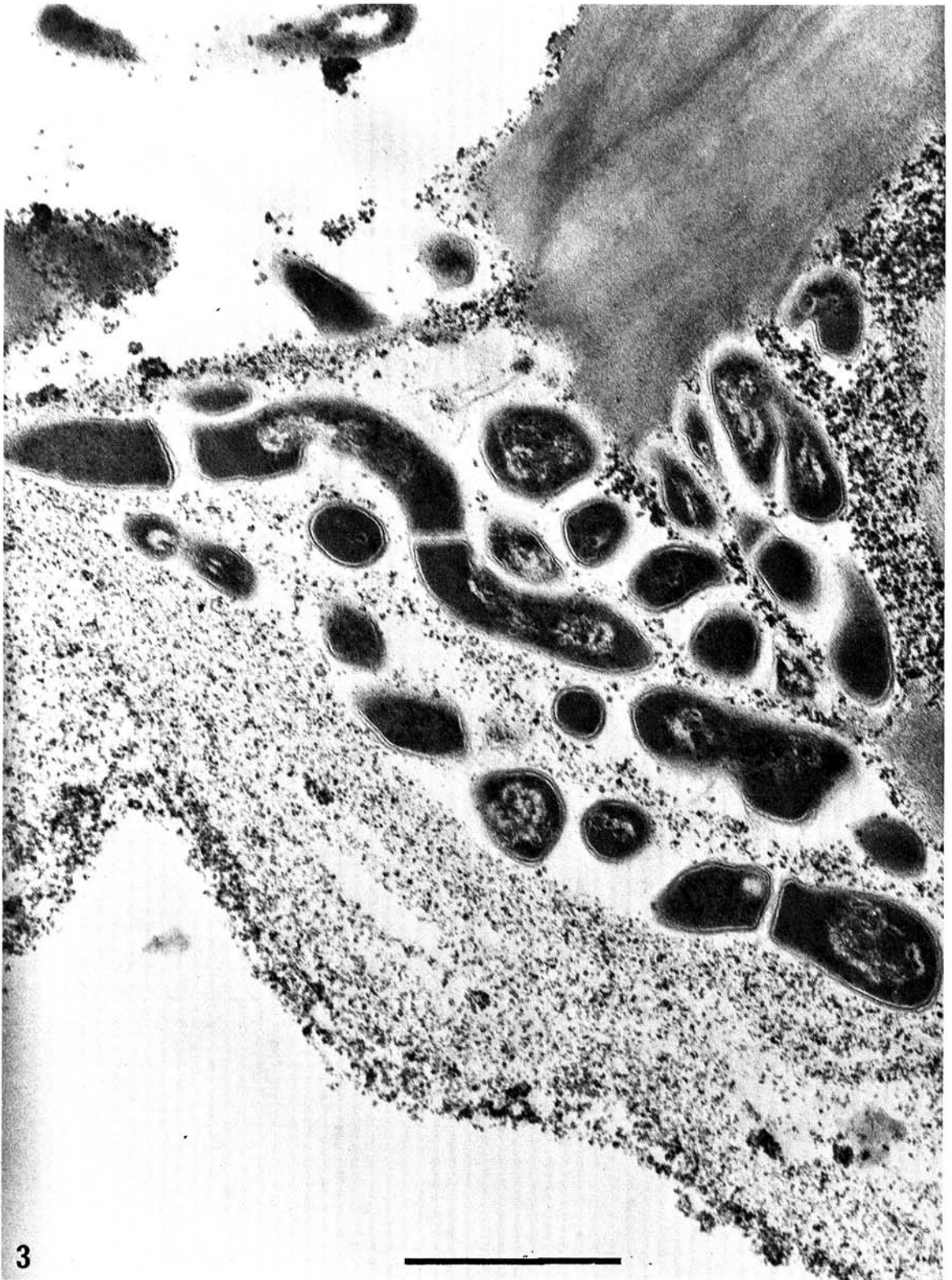
Fig. 3. Xylem cell of ratoon stunting diseased sudangrass hybrid showing coryneform bacterium. Note smooth cell wall, septa and fibrillar material in the central area. Bar is approximately 1 μm.

Fig. 4. Bacteria embedded in a matrix within a xylem cell of ratoon stunting diseased sudangrass hybrid. The layers are interpreted as 1) space between cytoplasm and membrane, 2) membrane, 3) space between membrane and cell wall, 4) cell wall, and 5) space between cell wall and matrix. Bar is approximately 1 μm.
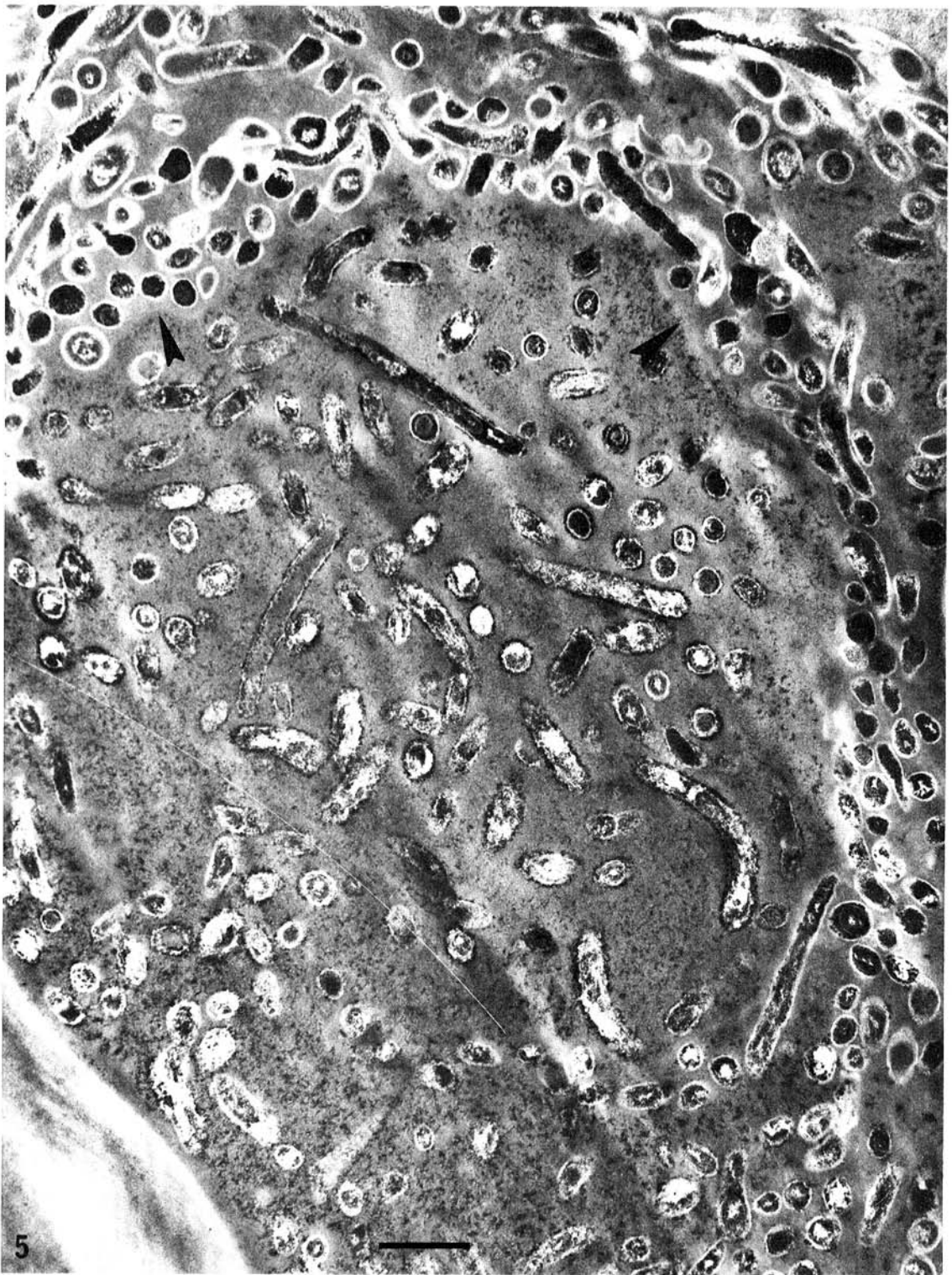
Fig. 5. Plugged xylem vessel of ratoon stunting diseased sudangrass hybrid containing bacterial cells embedded in homogeneous and granular matrices. Arrows show boundary of the two types of matrix. The bacterial cells in the granular matrix appear to be degenerating. Bar is approximately 1 μm.

vessel. In one xylem cell, the matrix varied from homogeneous to granular with a fairly defined boundary (Fig. 5).

In one Q28 hybrid plant, discolored bundles were dissected and embedded separately; many xylem cells were plugged, but no organism was apparent. These plugs appeared different in composition from those in the sudangrass hybrids. In cane, the plugs were more electron-opaque, and contained large spherical structures of unknown origin and composition (Fig. 6).

With light microscopy, the area in the sudangrass hybrid upright where the branch attached to the cutting was seen to contain a large network of vascular tissues. Many of the xylem vessels showed evidence of plugging. Plugs varied in density from a loose fibrillar material to an opaque crystalline substance. Some bacteriumlike bodies were observed more often in areas where the fibrillar material appeared to be well dispersed. Recognition of bacteria in situ with the light microscope was equivocal, because of the presence of many endogenous bodies in the same size-range. However, if the bodies were in fact bacteria, then their distribution in the base of the branch was extensive. Aerial roots from the same general area of the stem sett and branch showed xylem plugging, but no evidence of the bacteriumlike structures.

DISCUSSION.—Sugarcane appeared to be a poor choice of RSD material for finding the disease agent in thin-sections. Three criteria were used separately and in combination to choose diseased material for embedding: internal discoloration of vascular bundles in the node, infectivity of expressed sap, and the presence of a diagnostic bacterium in crude juice (3). Even all three combined did not always yield tissues that routinely contained bacteriumlike cells. The one sugarcane plant in which organisms were found in ultrasections did contain large numbers of the diagnostic bacterium in juice squeezed from part of the same node and showed extensive intense discoloration. But in similar tissues from other plants, no bodies were seen. The number of bodies present in the best sugarcane plant, however, was too small to allow a good description of the organism. Previous reports on the electron microscopy of sugarcane infected with RSD (8) showed a microorganism and termed it a small bacterium or rickettsia. Probably this indefinite description was caused also by a paucity of the bodies. Where only a few cells of the organism were scattered throughout many sections, characterization was difficult.

Although the reason for the apparent scarcity of the bodies in sugarcane is unknown, it may reflect the technical difficulties in sampling and sectioning canes. Xylem-plugging and discoloration of vascular bundles
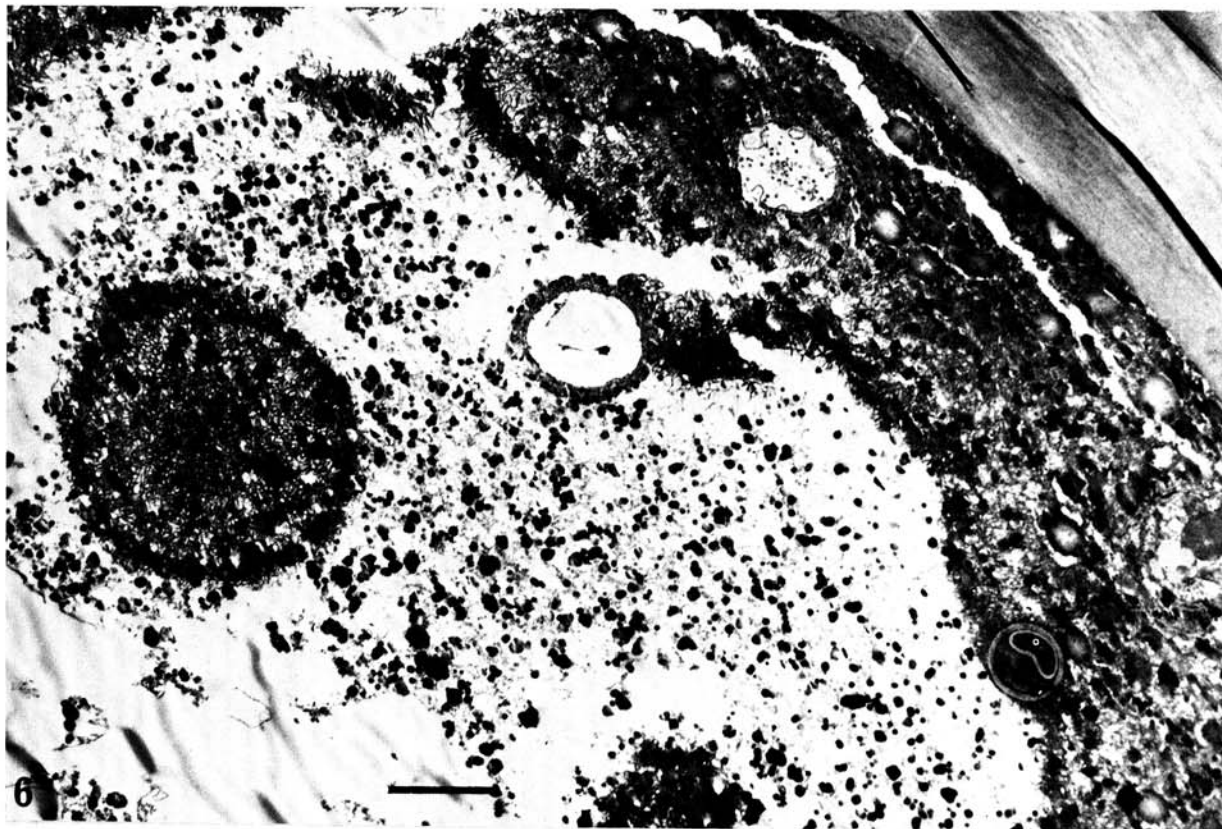

Fig. 6. Plugged xylem vessel of ratoon stunting diseased sugarcane variety Q28 showing electron dense material without discernible bacteria. Xylem vessel was taken from a disclosed vascular bundle. Bar is approximately 1 μm.
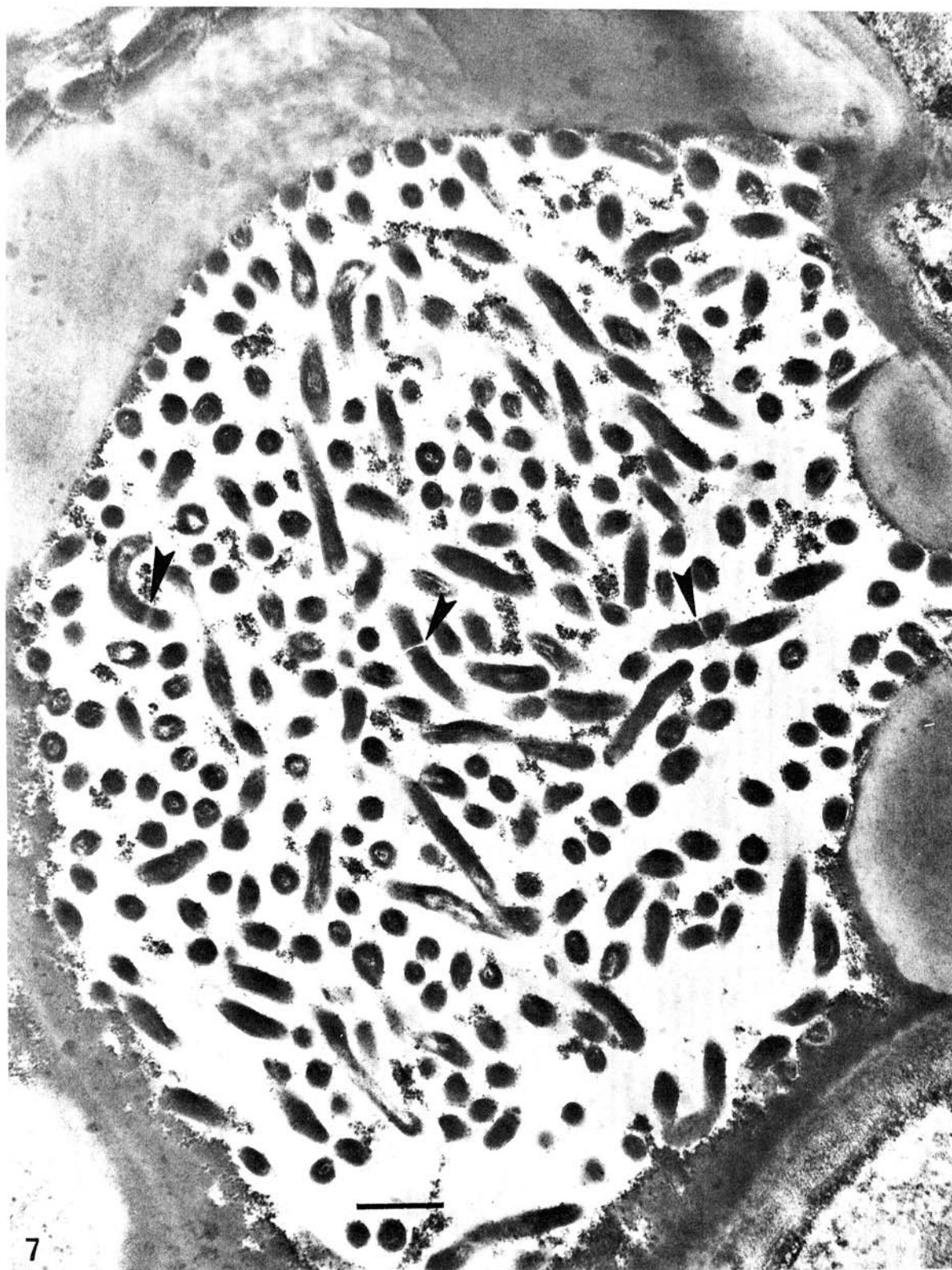
Fig. 7. Xylem vessel of ratoon stunting diseased sudangrass hybrid filled with bacteria. Note lack of binary fission and presence of septa. Bar is approximately 1 μm.

are typical symptoms of RSD, but their relation to the disease agent has not been shown. However, if we use visible discoloration of vascular bundles to estimate the distribution of the organism, then relatively few bundles would contain bacteria. In addition, the numerous fiber cells associated with the vascular bundles are difficult to section and necessitate trimming the block to three or less vessel cells. The combination of few infected cells and a small block of tissue hinders scanning by electron microscopy.

In contrast, the sundangrass hybrid upright was a good source of material for showing the presence and distribution of a suspected pathogen by electron microscopy. The organism was present in a high concentration in many xylem vessels. Also, tissue where the branch had been attached to the original cutting was easier to section than were nodes of sugarcane.

The presence of the organism in xylem cells confirms earlier reports. The organism seen by Maramorosch et al. (8) was in xylem cells. In previous studies (3), we observed by light microscopy a bacteriumlike organism in exudate from the cut stumps of RSD canes. The vessel habitat is suggested also by a recent report of Teakle et al. (10) who showed the presence of a coryneform-type bacterium in fibrovascular sap.

Xylem-contained microorganisms have recently been shown for two other plant diseases. A rickettsia-like organism was shown in xylem vessels of grape plants with Pierce's disease symptoms (4, 5). Phony peach disease also has a rickettsia-like organism associated with the xylem (6, 9). The organism described here differs from these rickettsia-like organisms in that the cell walls were not rippled.

Xylem-plugging, and the presence of a matrix in RSD plants, raises questions that presently cannot be answered. Is the matrix shown in Fig. 5 a stage of plugging? What is the relation of the bacteria to plugging? (Bacteria are not always seen in plugged cells; however, in cases where they are not present, the material is very dense and granular). Could the bacteria have been there at an earlier stage and distintegrated? [Disintegration of the bodies is suggested in Fig. 5 in the granular portion of the matrix. Even in the xylem vessels that were not plugged or did not have a definite matrix, usually particles were present, as shown in Figs. 3 and 7. These particles may be similar to the "finely granular staining substance" of Hopkins et al. (6) or to the "electron-dense ground substances" of Nyland et al. (9).]

Our characterization of the bacterium as a coryneform type is based on the presence of septa. The importance of septation as a distinguishing criterion was discussed by Komagata et al. (7) in the first of a series of papers on coryneform bacteria. In their studies they concluded that coryneform bacteria are distinguished from the true bacteria on the basis of cell division. The results of Teakle et al. (10) also show an association with RSD of an organism similar to a coryneform bacterium. Their organism in negatively stained preparations is somewhat smaller than the organism shown here in thin-sections. Nevertheless, the difference might have been a preparative artifact.

LITERATURE CITED

1. BENDA, G. T. A. 1971. Wilting and death in the ratoon stunting disease of sudangrass hybrid uprights. Am. Soc. Sugar Cane Technol. Proc. 1:39-47.
2. BENDA, G. T. A. 1973. The ratoon stunting disease and growth: Single-rooted plants of sugarcane and of sudangrass hybrid. Am. Soc. Sugar Cane Technol. Proc. 2:83-88.
3. GILLASPIE, A. G., JR., R. E. DAVIS, and J. F. WORLEY. 1973. Diagnosis of ratoon stunting disease based on the presence of a specific microorganism. Plant Dis. Rep. 57:987-990.
4. GOHEEN, A. C., G. NYLAND, and S. K. LOWE. 1973. Association of a rickettsia-like organism with Pierce's disease of grapevines and alfalfa dwarf and heat therapy of the disease in grapevines. Phytopathology 63:341-345.
5. HOPKINS, D. L., and H. H. MOLLENHAUER. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. Science 179:298-300.
6. HOPKINS, D. L., H. H. MOLLENHAUER, and W. J. FRENCH. 1973. Occurrence of a rickettsia-like bacterium in the xylem of peach trees with phony disease. Phytopathology 63:1422-1423.
7. KOMAGATA, K., K. YAMADA, and H. OGAWA. 1969. Taxonomic studies on coryneform bacteria: I. Division of bacterial cells. J. Gen. Appl. Microbiol. 15:243-259.
8. MARAMOROSCH, K., B. PLAVSIC-BANJAC, J. BIRD, and L. J. LIU. 1973. Electron microscopy of ratoon stunted sugar cane: microorganisms in xylem. Phytopathol. Z. 77:270-273.
9. NYLAND, G., A. C. GOHEEN, S. K. LOWE, and H. C. KIRKPATRICK. 1973. The ultrastructure of a rickettsia-like organism from a peach tree affected with phony disease. Phytopathology 63:1275-1278.
10. TEAKLE, D. S., P. M. SMITH, and D. R. L. STEINDL. 1973. Association of a small coryneform bacterium with the ratoon stunting disease of sugar-cane. Aust. J. Agric. Res. 24:869-874.