Microcolonies of the Bacterium Associated with Ratoon Stunting Disease Found in Sugarcane Xylem Matrix

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ABSTRACT

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Microcolonies of a filamentous, branched organism were observed by phase-contrast and interference-contrast microscopy in vascular extracts from internodes of sugarcane with ratoon stunting disease. The microcolonies were present in a matrix extracted from the xylem vessels of internodes by negative pressure. Matrices and microcolonies were not found in sugarcane known to be free of the disease. Electron microscopy revealed branched chains of cells with septa and mesosomes. The individual cells of the filaments were characteristic of the previously described coryneform bacterium associated with the disease. Vascular extracts from one- or two-node cuttings of diseased cane did not reveal matrices or microcolonies but did contain the individual cells or short branched chains of the organism. The filamentous spiderlike morphology of the microcolonies and true branching suggest a relation to the Actinomycetales.

Ratoon stunting disease (RSD) of sugarcane was recognized in Queensland, Australia in 1944 and was presumed to be caused by a virus (8). A coryneform bacterium was associated with the disease in 1973 (4, 10). Subsequently many workers have confirmed this association (1, 2, 3, 7).

Several methods to obtain the bacterium from ratoon stunting diseased sugarcane have been reported: fibrovascular extraction (3, 10), root pressure exudation (4), juice expression (3, 4), and diffusion from tissue (1, 9). The bacterium obtained by these methods always was a pleomorphic, sometimes septate, mesosome-containing rod with a smooth cell wall, and approximately 0.25 × 1 - 3.5 μm.

Because the bacterium has not yet been isolated and cultured, its taxonomic position has not been determined. However, its morphology in vivo indicated that it might belong to the coryneform group of bacteria, as first suggested by Teakle et al. (10).

This paper reports our observations on the in vivo occurrence of the RSD-associated bacterium in microcolonies which resemble an actinomycete in their habit of growth.

MATERIALS AND METHODS

Single-node cuttings of ratoon stunting diseased and healthy sugarcane cultivar L 62-96 were planted in the greenhouse in a potting mix of sand, peat moss, and perlite (3:2:1, v/v) in galvanized metal flats. Criteria for RSD were the RSD-associated bacterium and the internal symptom of red-colored vascular bundles in the nodes when the stalk was split. These plants were grown for approximately 6 mo. Temperature during the growth period varied between 20 and 38 C.

Vascular extracts were collected by a modification of the method of Teakle et al. (10). Basal internodal or nodal pieces 3-4 cm in length were inserted into the neck of a vacuum flask and sealed in with Permagum (Virginia Chemicals Inc., W. Norfolk, VA 23703). Deionized water was dripped onto the upper cut surface from a buret or pipet and a water aspirator provided a vacuum in the flask. The vascular extract collected in the flask was centrifuged for 20 min at 3,000 *g*.

The pellets were resuspended in a few drops of water for microscopic examinations. Light microscopic examinations were carried out with a Leitz Ortholux II microscope equipped with interference- and phase-contrast optics and an Orthomat W camera. Transmission electron microscopic examinations were made with a Hitachi HU-11-A electron microscope. The suspensions were dried at room temperature on grids of carbon-coated Parlodion. Bacteria were negatively stained for 15 sec with a 1.0% solution of sodium phosphotungstate (pH 6.3).

RESULTS

Cylindrical matrices resembling extruded toothpaste consistently were seen in vascular extracts of internodes of diseased cane when viewed with interference contrast optics at magnifications of ×250 or ×625 (Fig. 1). Matrices varied greatly in length and were from 10-110 μm in diameter, most being 50-60 μm in diameter, and had either a granular (Fig. 1-A) or a homogenous (Fig. 1-B) appearance.

Many circular or spiderlike colonies of 5-20 μm in diameter were found in the matrices (Fig. 1). These were revealed as microcolonies of a filamentous branched organism under phase- and interference-contrast optics at ×1,562 (Fig. 2-A). Individual cells presumed to be fragments from the microcolonies also were observed. These were indistinguishable from the previously

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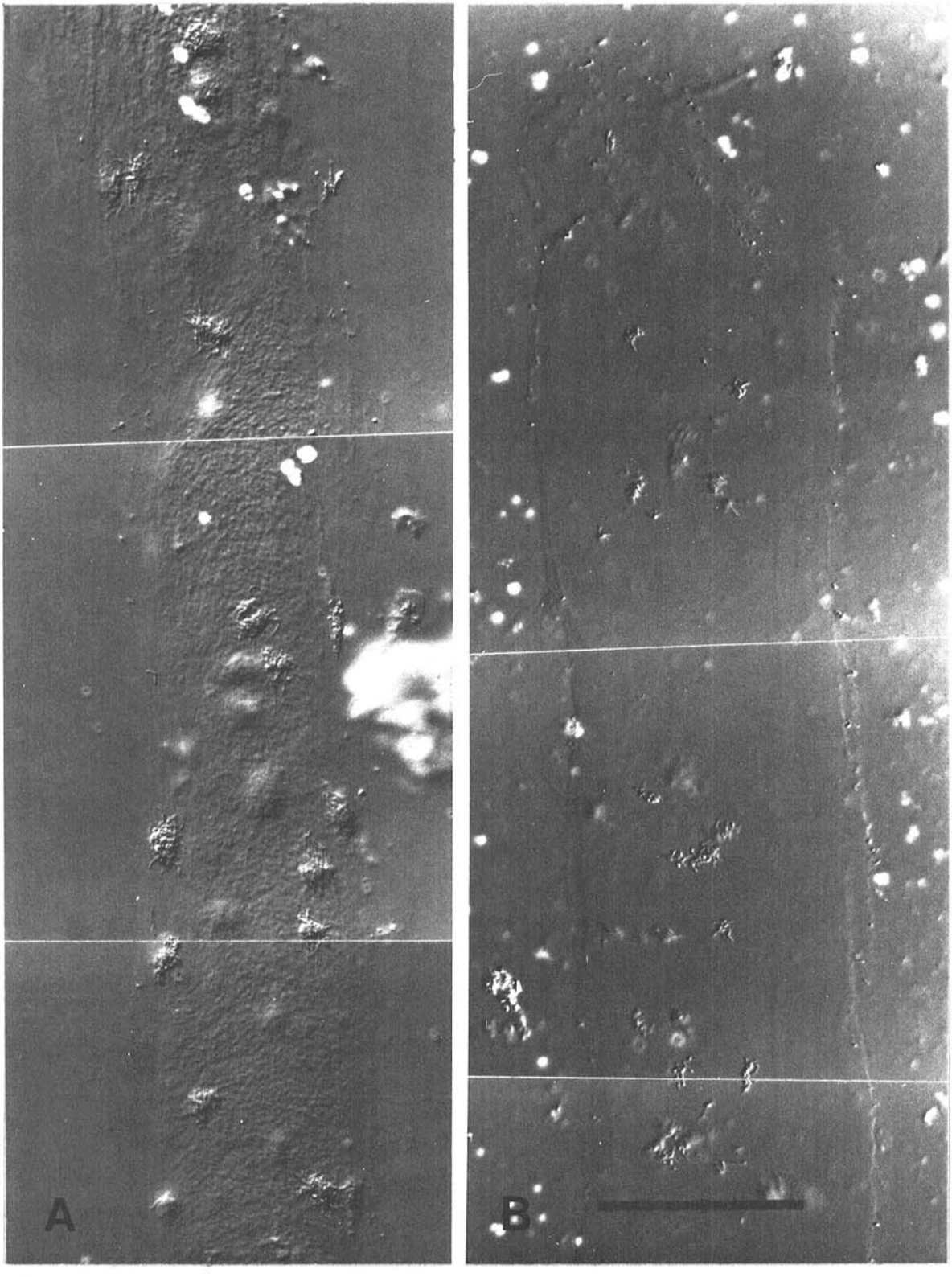

Fig. 1-(A, B). Interference contrast micrographs of the matrix extracted from xylem vessels of ratoon stunting diseased sugarcane internodes. Note spiderlike microcolonies in the **A)** granular and **B)** homogeneous forms of the matrix. Bar is approximately 50 μm.

detected unicellular forms of the RSD-associated bacterium.

Occasionally microcolonies were not associated with a matrix but were observed free in the vascular extracts (Fig. 2-B). These may have been dislodged from the matrix during the extraction process. The microcolonies were characterized by a dense center and a branching, filamentous network of growth toward the margins (Fig. 1-A, 2-A, B). Individual branching filaments (Fig. 2-C) also occurred free in the vascular extracts.

Vascular extractions through one- or two-node cuttings of ratoon stunting-diseased cane did not reveal identifiable matrices or microcolonies, although numerous unicellular and short filamentous forms were observed. Vascular extracts from healthy cane revealed no matrices, microcolonies, or unicellular forms.

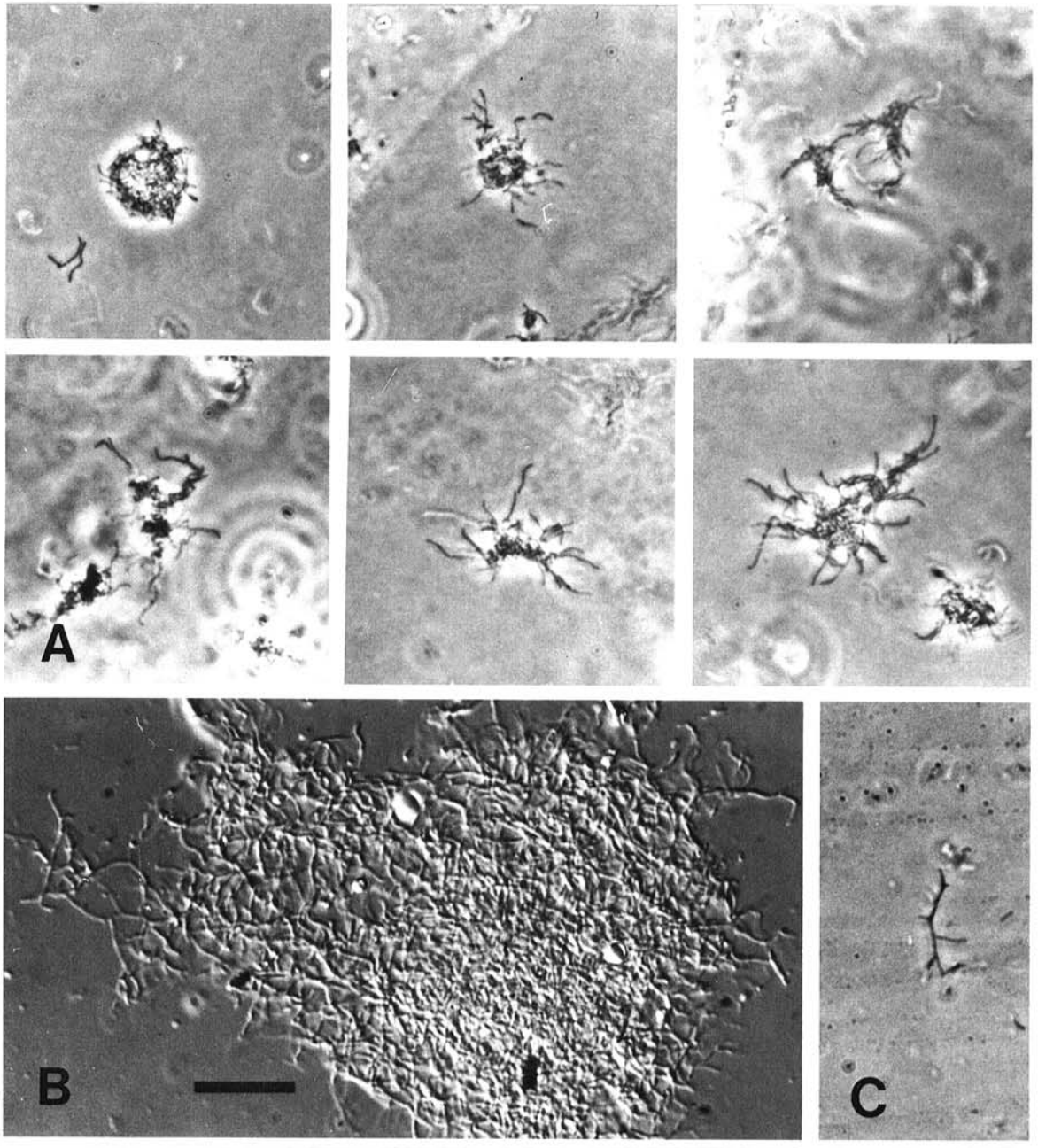

Fig. 2-(A, B, C). A) Phase-contrast micrographs of microcolonies of the sugarcane ratoon stunting disease-associated organism present in the matrix. **B)** Interference-contrast micrograph of a free microcolony. Note dense center and branching network toward the margin. **C)** Phase-contrast micrograph of short branching filament. Bar is approximately 10 μm.

Electron microscopic examinations revealed that the branching filaments were septate, contained mesosomes, and had a smooth and thin cell wall surrounding a cell membrane (Fig. 3). Some connections between adjacent cells appeared very weak which suggested that the filaments might easily fragment to single cells (Fig. 4-A). The branching often occurred adjacent to the septum and the lateral growth of the branches frequently occurred at right angles to the somata (Fig. 4-B).

Occasionally RSD-associated bacteria with swollen

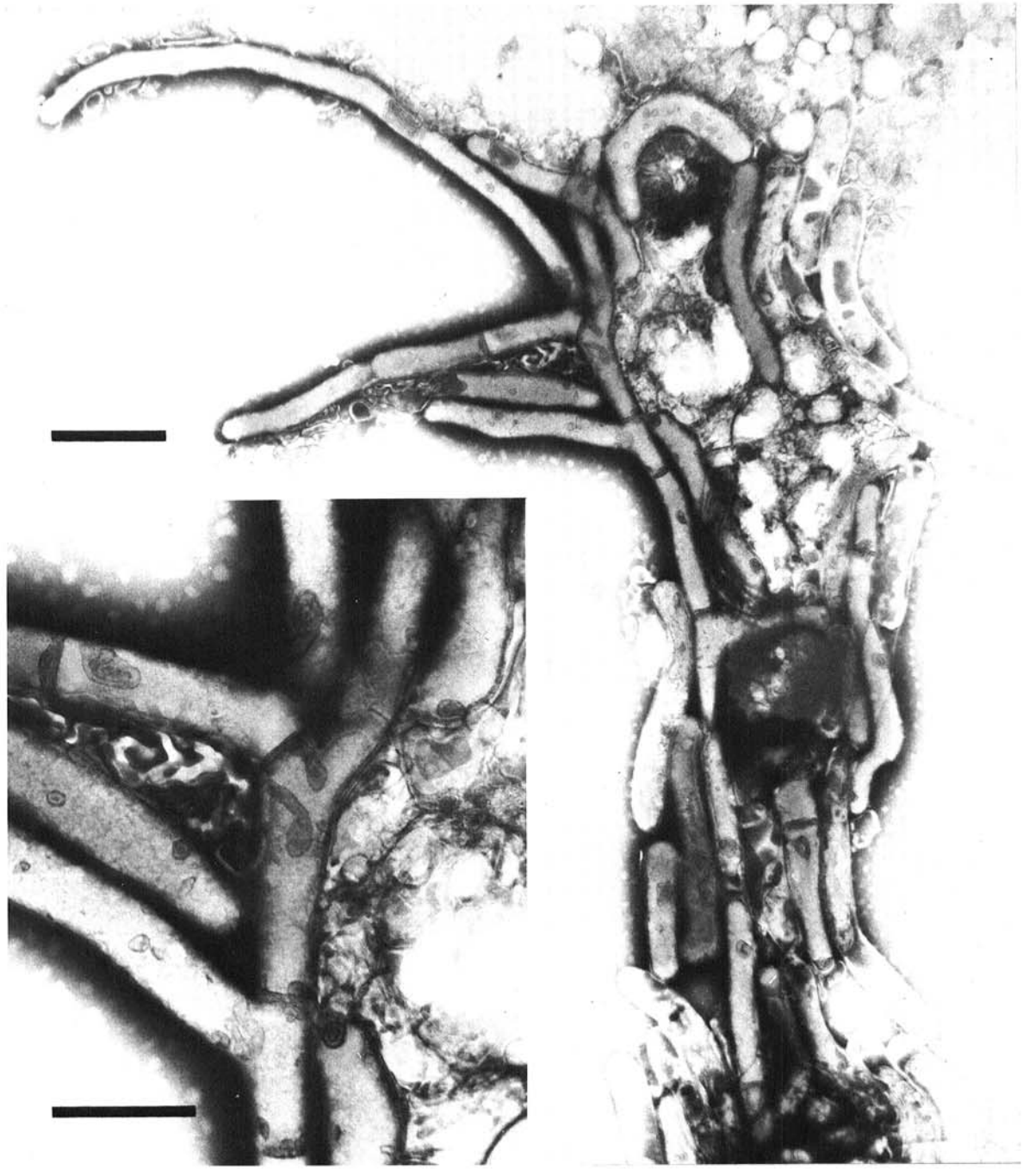

Fig. 3. Electron micrograph of branching filaments of the sugarcane ratoon stunting disease-associated organism. Unicellular forms also are apparent. Bar is approximately 1 μm. Note branching, septations, and mesosomes which are most apparent in the inset. Bar is approximately 0.5 μm.

terminal or subterminal portions were present either in the matrix or free (Fig. 5). The swollen portion had a diameter of approximately 0.5-1.0 μm. The swollen bacteria appeared to be the RSD-associated bacterium on the basis of septa, mesosomes, and the smooth cell walls (Fig. 5-B, C, D). Although we have frequently seen this peculiar structure, no interpretations are provided at this time.

DISCUSSION

The matrices and associated microcolonies have not been detected in vascular extracts. This is probably because of the practice of making extracts through a node, instead of an internode. Vascular bundles at the nodes lose their straight parallel arrangement, branch, and enter buds, leaf sheaths, and root primordia. This

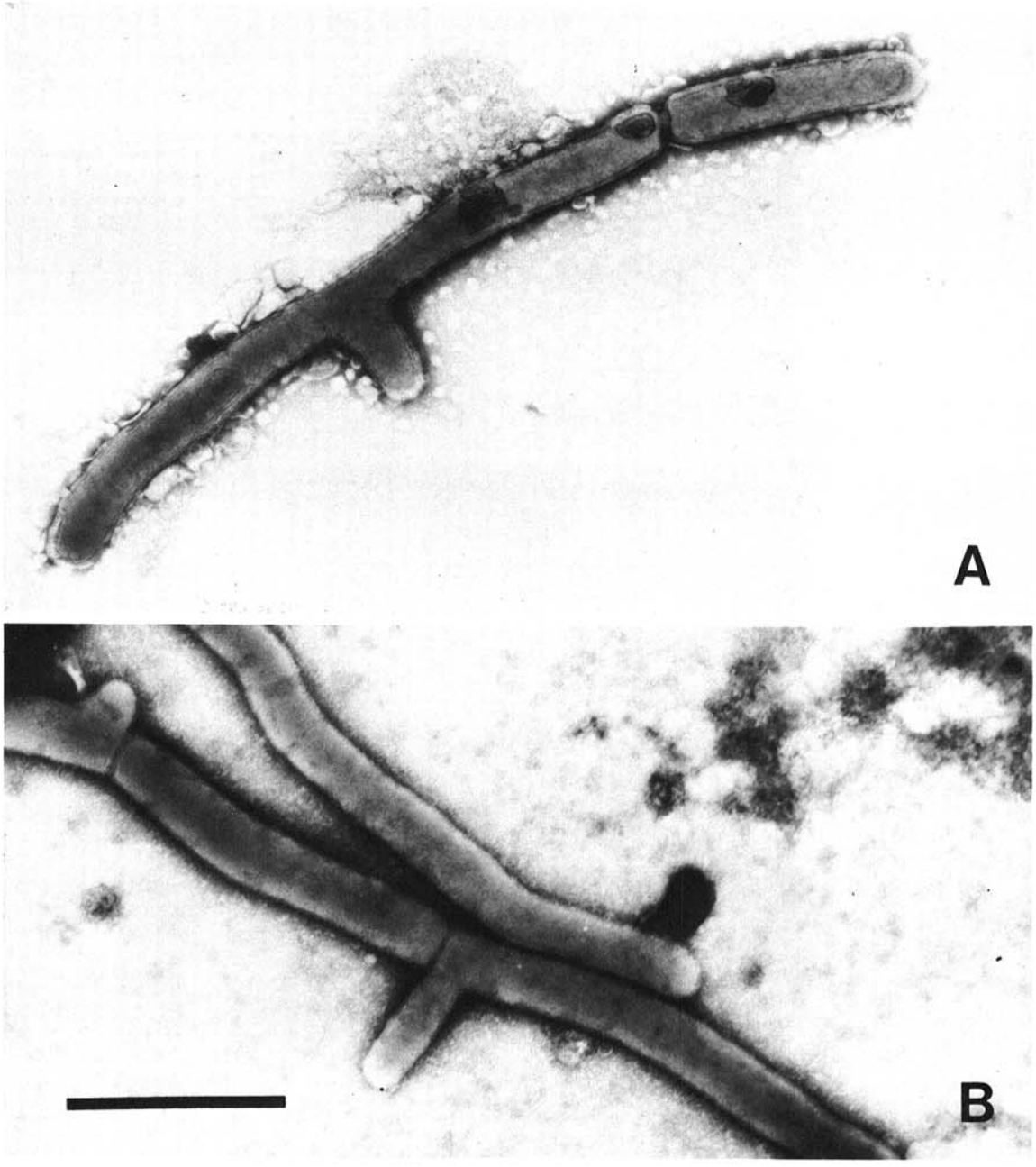

Fig. 4-(A, B). Electron micrographs of the sugarcane ratoon stunting disease-associated organism depicting **A)** branching, smooth multilayered cell wall, and potential fragmentation site between cells. **B)** Branch formation occurring adjacent to septum. Bar is approximately 1 μm.

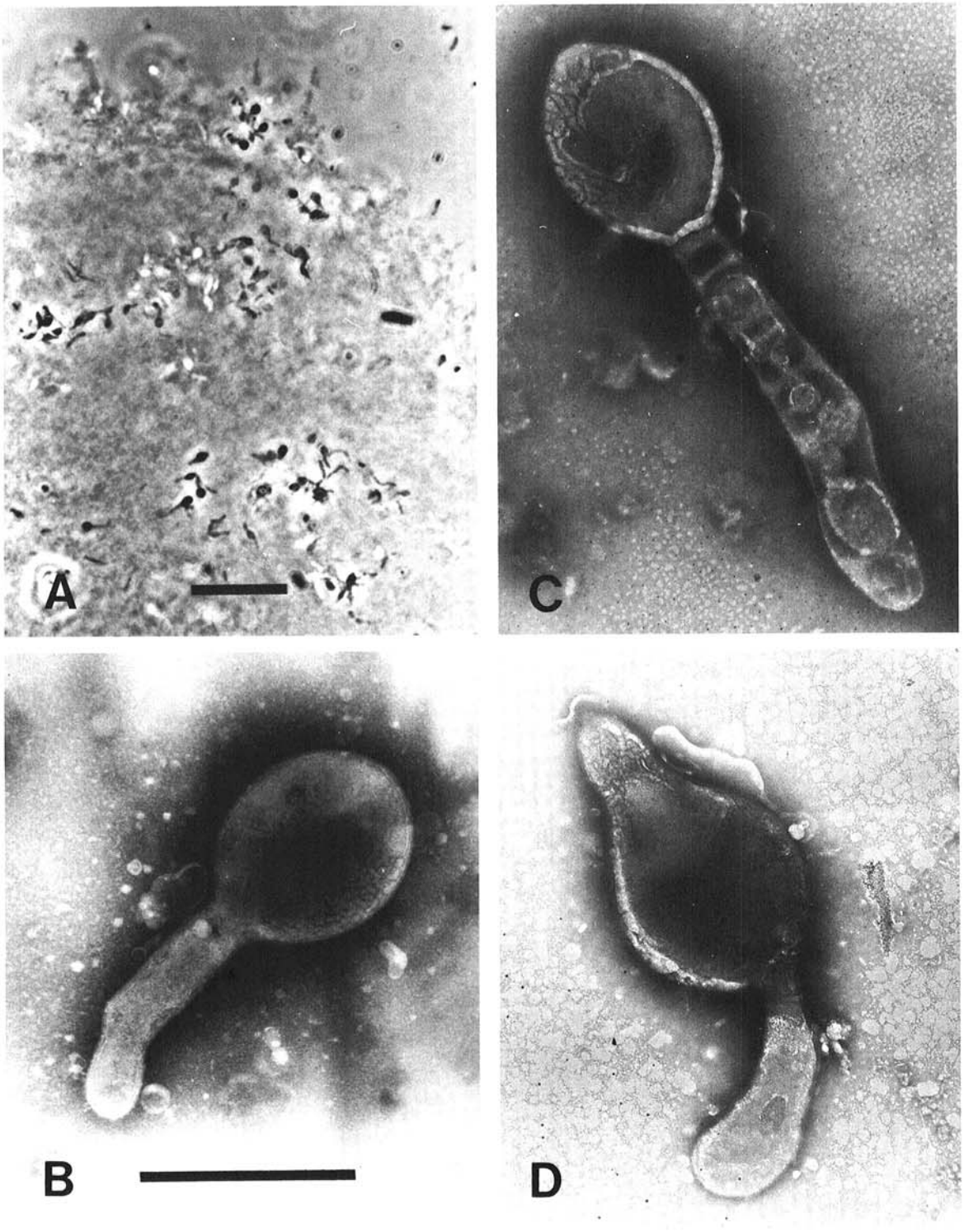

Fig. 5-(A, B, C, D). Swollen forms of the sugarcane ratoon stunting disease-associated organism. **A)** Phase-contrast micrograph. Bar is approximately 10 μm. **(B-D)** Transmission electron micrographs of one- and two-celled swollen forms. Note septa and mesosomes. Bar is approximately 1 μm.

results in greater resistance to flow of water through nodes than through internodes (Kao and Damann, *unpublished*) and presumably retards release of the matrix. The origin and role of the matrix in the disease have not been determined. It could be a host reaction product and source of nutrition for the bacterium or it could be synthesized by the bacterium. The microcolonies appearing to be on the surface of the matrix is an optical artifact of interference contrast microscopy.

Because the cylindrical matrices had a diameter (10-110 μm) similar to the xylem vessels (12-70 μm) they probably were washed out of the xylem. Furthermore, recent reports based on thin sections of RSD-infected sugarcane (5) and sudangrass (11) indicate that xylem vessels contain a matrix around the RSD-associated bacteria. One of these reports also recognized granular and homogeneous forms of the matrix (11). These reports plus the morphological evidence convinced us that the bacterial colonies embedded in the matrices were the RSD-associated bacterium.

Teakle et al. (10) reported the bacterium as being coryneform. The criteria were: club-shape, presence of septa, and snapping or bending type division. In addition to these characteristics, we have demonstrated the formation of microcolonies containing filamentous branching cells which are characteristic of Actinomycetales.

Coryneform bacteria do not exhibit true branching, however, when branching does appear, it is termed "transient branching" because it is formed by temporary attachment of already divided cells (6). Our electron microscopic observations demonstrate that true branching does exist in the RSD-associated organism (Fig. 3, 4). We conclude that the microcolonies and swollen forms found in matrices of ratoon stunting diseased cultivar L 62-96 constitute additional stages in the growth cycle of the RSD-associated bacterium and suggest its relation to the Actinomycetales.

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