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Scanning electron and light microscopy of infection and symptom development in tomato leaves infected with *Pseudomonas tomato*

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Pseudomonas tomato infection of tomato leaves was followed by scanning electron and light microscopy. The primary infection sites were the stomata and the bases of the leaf trichomes from which bacterial masses burst out at later stages of disease development. During incubation the bacteria disappeared from the surface of the leaves of the susceptible variety and became located in the intercellular spaces. Necrosis was first observed microscopically 100 h after inoculation. Bacteria were detected in infected tissue but not in necrotic tissue. Lesions increased in numbers and in size until the leaves were totally infected with bacteria. Bacteria disappeared from the leaves of the resistant tomato cultivar within 140 h of inoculation.

INTRODUCTION

Pseudomonas tomato (Okabe) Alstatt [*1*] causes damage to crops of tomato in Israel [*30*]. The pathogen is specific to tomato and produces typical leaf symptoms on its host, e.g. small (2 to 3 mm diameter) dark brown to black specks with a distinct yellow halo. In heavily infected plants these lesions can coalesce to form irregular dark brown necrotic patches [*4, 19, 28*]. There is a period of 5 to 8 days between inoculation and the appearance of visible symptoms [*3, 6, 18, 22*].

Ultrastructural studies of the interaction between phytopathogenic bacteria and plant cells have been reported for *Xanthomonas malvacearum* in cotton [*7*], *X. campestris* in cabbage [*27*], *Pseudomonas pisi* and *P. solanacearum* in tobacco [*9, 24*], *P. tabaci* in tobacco callus [*11*], *P. phaseolicola, P. syringae* and *P. tomato* in bean [*5, 25, 26*] and *Erwinia amylovora* in apple [*12*]. These studies revealed that incompatible bacteria become attached to plant cell walls and are enmeshed by fibrilar and granular structures. Most investigators noted that the compatible pathogen multiplied freely in the intercellular spaces of the host plant tissue. However, tissue necrosis caused by *P. tomato* has as yet not been monitored at the ultrastructural level. The purpose of this work was to carry out such a study and follow the multiplication of *P. tomato* and the development of lesions, in both susceptible and resistant tomato plants.

MATERIALS AND METHODS

Organisms and growth conditions

Pseudomonas tomato (WT-1), isolated from infected tomato plants in Israel [*3, 4*],

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was kept at room temperature on King-B agar slants [*13*] and transferred to a fresh medium every month.

Cultures used as inoculum were grown on yeast peptone medium (YP) [*21*] in a shaker bath (100 strokes min^{-1}) at 30 °C for 24 h. The cultures were centrifuged at 10 000 *g* for 10 min and were resuspended in saline (NaCl, 0·85 g l^{-1}) to give 0·3 absorbance units at 540 nm in a Junior II Coleman spectrophotometer, corresponding to 10^9 cells ml^{-1}. Bacteria were counted by the dilution plate method [*14*] on a modified King-B medium [*13*] supplemented (μg ml^{-1}) with basic fuchsin, 9; cycloheximide, 9; 2,3,5-triphenyl-tetrazolium-chloride, 1·4; nitrofurantoin, 10; and nalidixic acid, 23 [*10*]. On this medium neither gram positive bacteria nor fungi could grow. Colonies of the pathogen differed morphologically from other fluorescent colonies of saprophytes. They developed at a slower rate, had wrinkled edges and were oxidase negative. Cultures were routinely tested for pathogenicity every month.

Tomato plants (*Lycopersicon esculentum*) of the resistant cultivar Rehovet-13 [*29*] and the highly susceptible cultivar VF-198 [*30*] were grown in vermiculite and peat 50 : 50 (v/v) in a phytotron at 27 °C (day) and 21 °C (night) under a 14-h day and were irrigated with Hoagland's solution. Only plants with 4 to 6 true leaves were used for inoculation.

Bacterial inoculation

Eight tomato plants of each cultivar were inoculated by one of the following methods: (a) plants were preincubated under periodic mist (5 s mist every 30 min) [*6*] for 24 h, sprayed with 10^6 cells ml^{-1} and incubated in a mist chamber at approximately 100% r.h. at 25 °C [*3*]; (b) 20 leaflets were surface-sterilized with commercial 3% NaOCl for 3 min. The samples were washed 5 times with sterile saline to remove traces of hypochlorite and then placed (3 leaflets per Petri dish) on 0·5% water agar. Several areas of the lower surface of each leaflet were inoculated with a suspension of *P. tomato* cells (10^4 ml^{-1}) and incubated in a growth chamber under constant light (12 000 lux) at 25±2 °C.

Determination of bacterial populations

Triplicate samples, each of 5 cm^2 in area, were cut from young infected leaves and surface sterilized by immersion in 3% NaOCl for 5 min. The sterilized samples were washed with sterile water and homogenized in 20 ml of sterile saline in an Omni-mixer (Sorvall) maintained in an ice bath. The homogenates were diluted serially 10-fold and 0·1 ml portions were spread with a glass rod [*14*] on the surface of modified King-B medium plates [*13*]. The inoculated plates were incubated for 5 days at 25±2 °C.

Scanning Electron Microscopy (SEM)

Leaves of plants from different inoculation treatments were examined by SEM at 24 h intervals during the first 6 days after inoculation. Ten samples from each treatment were prepared for observation as described elsewhere [*3, 6*].

Light Microscopy

Twenty-four hours after inoculation, 10 leaves from each cultivar, were immersed for 5 min in a boiling solution composed of 31 ml glycerol, 16 ml lactic acid, 20 g crystalline phenol, 20 ml water and 125 ml absolute ethanol. The leaves were then washed with absolute ethanol, transferred to a boiling solution of 78 g KOH in 68 ml water for 1 min. A longer treatment of the leaves resulted in their complete dissolution. The treated leaves were washed further with absolute ethanol and then boiled for 4 min in a mixture of 30 g chloral-hydrate, 20 ml lactic acid, 5 ml absolute ethanol and 50 ml of 1% Aniline blue. The stained samples were finally washed with absolute ethanol [*2*].

RESULTS AND DISCUSSION

From the pioneering investigations of Rolfs [*20*] to the present [*8*] it is commonly agreed that the natural openings of plants (e.g. stomata, lenticels, hydathodes, trichomes) and wounds are a significant site of entry for pathogenic bacteria. After penetrating the tissue, the bacteria rapidly multiply in the intercellular spaces and this finally results in tissue necrosis [*15, 23*]. In consequence, most studies using transmission electron microscopy have concentrated on the events taking place inside the plant tissue rather than on the leaf surface.

In our studies we found no bacterial cells on uninoculated leaves or in their tissue (Plates 1 and 3). Ten minutes after inoculation with *P. tomato*, using 10^6 cells ml^{-1}, only a few bacteria were observed on the leaf surface (Plate 2), and 24 h later no bacteria were visible. However, microcolonies of the pathogen could be observed, using the light microscope, in the stomata, sub-stomatal chamber and in the intercellular spaces below the epidermal cells (Plates 4 to 6). These observations of invasion sites are in accordance with the hypothesis that even very small numbers of pathogenic bacteria at the right site are sufficient to induce infection [*8*].

Forty-eight hours after inoculation, randomly dispersed bacteria were observed all over the leaf surface (Plate 7). In specific areas such as stomata and trichome bases extensive multiplication occurred (Plates 8 and 9) with the areas surrounding these sites becoming full of bacteria (Plate 9). When the leaf samples were mechanically abraded, large numbers of *P. tomato* were observed inside the tissue (Plate 10). In addition, bacteria seemed to burst out of the stomata (Plate 11). A similar phenomenon was first observed in peach leaves infected with *X. pruni* [*17*]. These findings are in agreement with those of Schneider & Grogan [*23*] who suggested that the tomato leaf trichomes are a source of resident inoculum, and the target sites for infection by *P. tomato*.

Seventy-two hours after inoculation, bacteria were concentrated mainly at broken trichome bases (Plates 12 and 13), and 100 h after, necrotic areas became apparent at these sites (Plate 14). The border line between necrotic area (speck) and apparently healthy tissue was sharp (Plate 15). All the tissues bordering the lesions, including the stomata, were flooded with bacteria, but the necrotic areas were free of bacterial cells. This suggests the possibility that the pathogen dies when the host cells die.

After 5 days, many sites on the inoculated leaves were covered with streams of bacteria (Plate 16), and it was sometimes difficult to identify the morphological

details of the leaf surface because of the thickness of the bacterial mass. Even the usually clean necrotic sites were not detected (Plate 17). Trichome bases were destroyed (Plate 18) and the trichomes became detached.

After 140 h necrosis was almost total and any areas which did not collapse were covered with masses of the pathogen (Plate 19).

A different pattern of infection was observed in the resistant cultivar Rehovot 13 inoculated with *P. tomato*. Following inoculation, bacteria slowly disappeared with time. After 100 h only a few bacteria were observed (Plate 20), and after 140 h they could only be found with difficulty (Plate 21).

These phenomena led us to the conclusion that the resistant cultivar inhibited, in a yet unknown way, the multiplication of *P. tomato* both inside and outside the leaves, whereas the susceptible cultivar favoured the multiplication of the pathogen.

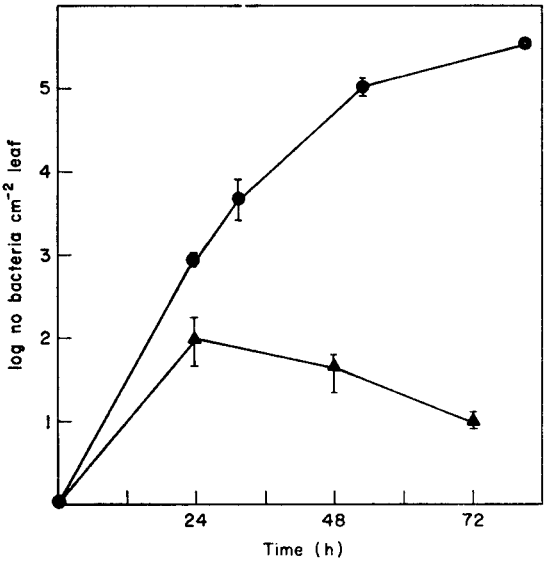

FIG. 1. Recovery of *Pseudomonas tomato* (W T–1) in susceptible VF 198 (●) and resistant Rehovot 13 (▲) tomato leaves. Points and bars represent mean and standard error of 3 replicates.

Bacterial counts inside the leaf increased in the susceptible cultivar and decreased in the resistant one (Fig. 1). A similar phenomenon was reported by Mew & Kennedy on the surface of soybean leaves infected with *P. glycinea* [*16*]. These findings fill the gap in our knowledge, which existed, about events between inoculation by *P. tomato* and the appearance of visible symptoms on the tomato leaf.

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Abbreviations: B, bacteria; T, trichome; S, stomata; N, necrotic tissue; H, apparently healthy tissue.

PLATE 1. SEM of the surface of susceptible tomato leaf before inoculation. ×2400.

PLATE 2. SEM of *P. tomato* cells on the surface of a susceptible tomato leaf immediately after inoculation. ×2400.

PLATE 3. Light micrographs of non-inoculated tomato stomata. ×400.

PLATES 4 to 6. Light micrographs of microcolonies of (arrows) *P. tomato* in the stomata (Plate 4), sub-stomatal chamber (Plate 5) and in the intercellular spaces below the epidermal cells (Plate 6), 24 h after inoculation. ×1000.

PLATE 7. SEM of *P. tomato* cells 48 h after inoculation. ×1300.

PLATES 8 and 9. Multiplication of *P. tomato* 48 h after inoculation in stomata (Plate 8, ×2400) and trichome base (Plate 9, ×1000).

PLATE 10. Mechanical crack in the leaf tissue 48 h after inoculation. ×2400.

PLATE 11. *P. tomato* cells exude from tomato stomata after 48 h. ×4700.

PLATES 12 and 13. *P. tomato* in detached (Plate 12, ×1300) and broken trichomes (Plate 13, ×500) 72 h after inoculation.

PLATE 14. First necrosis near trichome base 100 h after inoculation. ×330.

PLATE 15. Border line between necrotic area and apparently healthy tissue. ×1300.

PLATE 16. Typical stream of *P. tomato* on the leaf surface 120 h after inoculation. ×2200.

PLATE 17. Necrotic and non-necrotic areas under layers of bacteria. (arrows) 120 h after inoculation. ×2400.

PLATE 18. Destruction of tomato trichome base after 120 h. ×3600.

PLATE 19. Apparently healthy tissue which showed no signs of collapse after 120 h. ×2700.

PLATES 20 and 21. Pattern of infection by *P. tomato* in a resistant cultivar "Rehovot 13" after 100 h (Plate 20, ×2700) and 140 h (Plate 21, ×2400).

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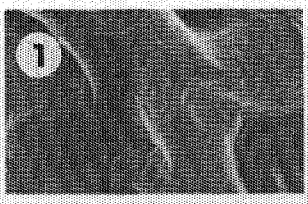
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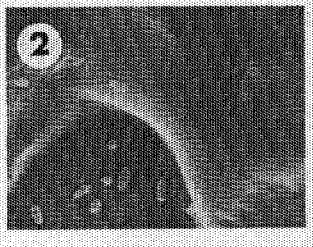
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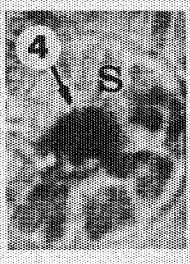
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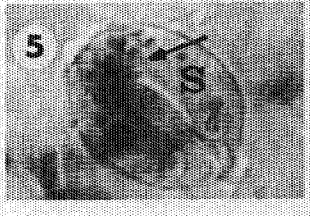
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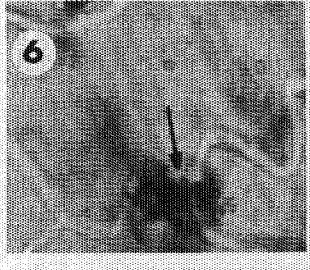
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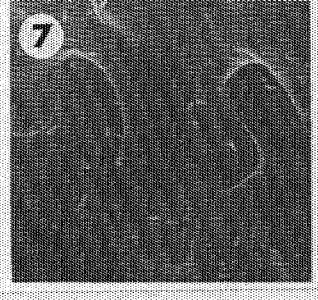
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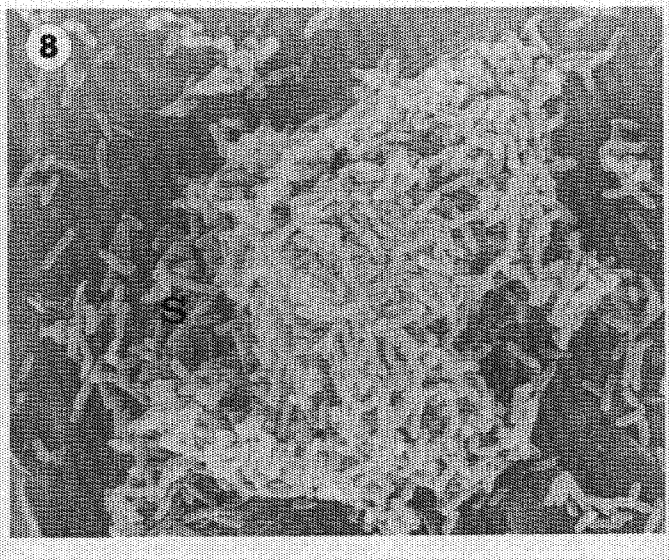
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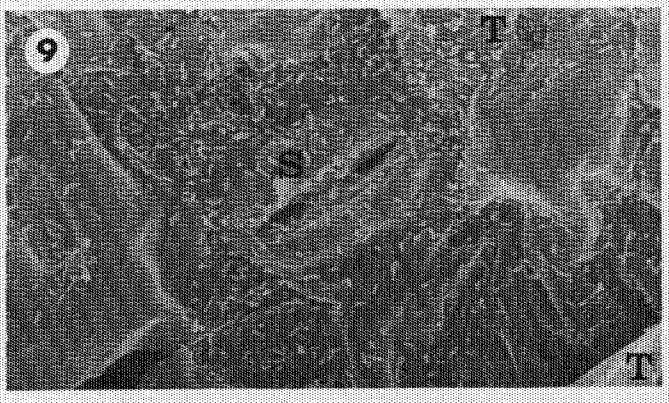
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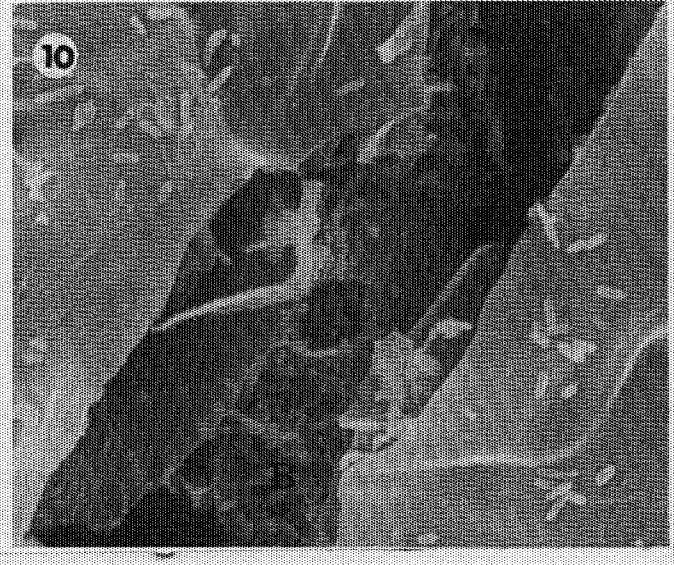
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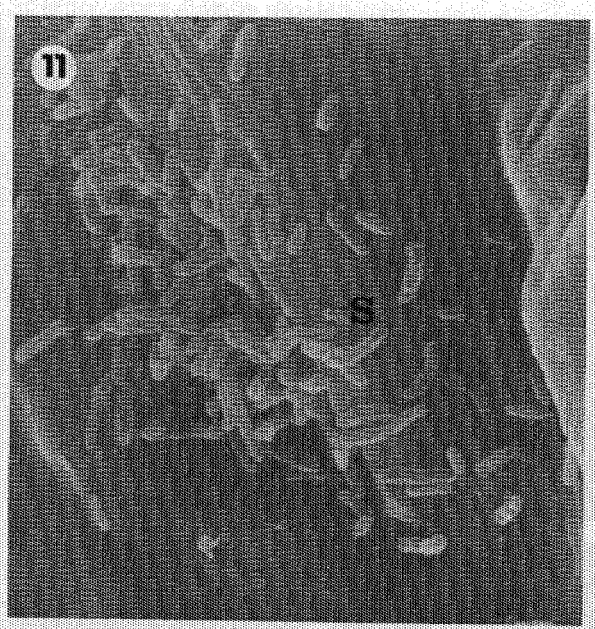
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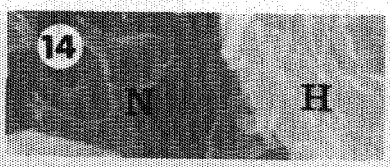
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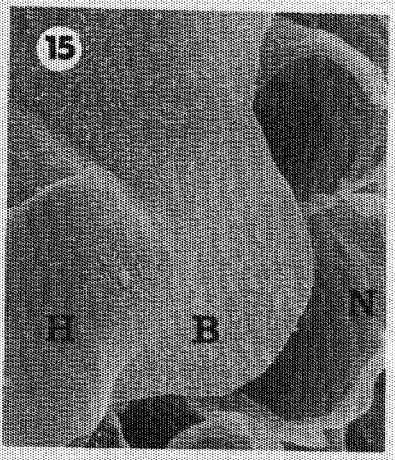
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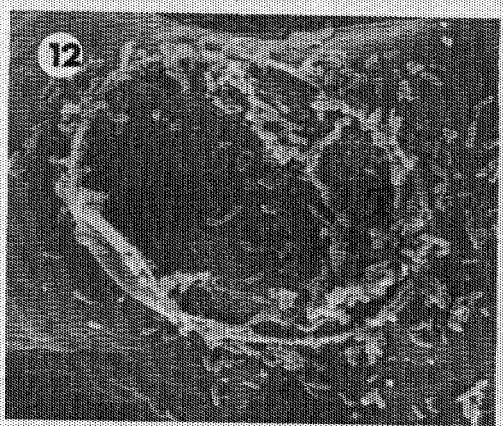
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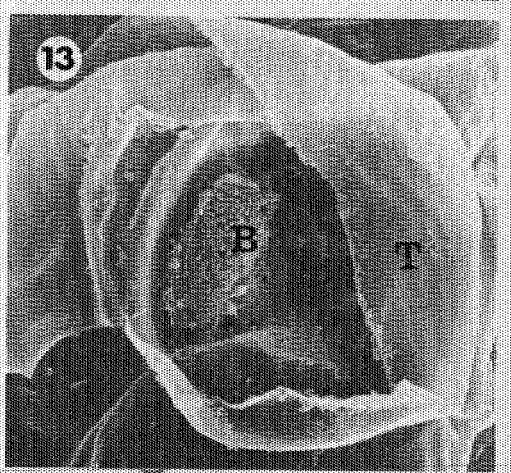
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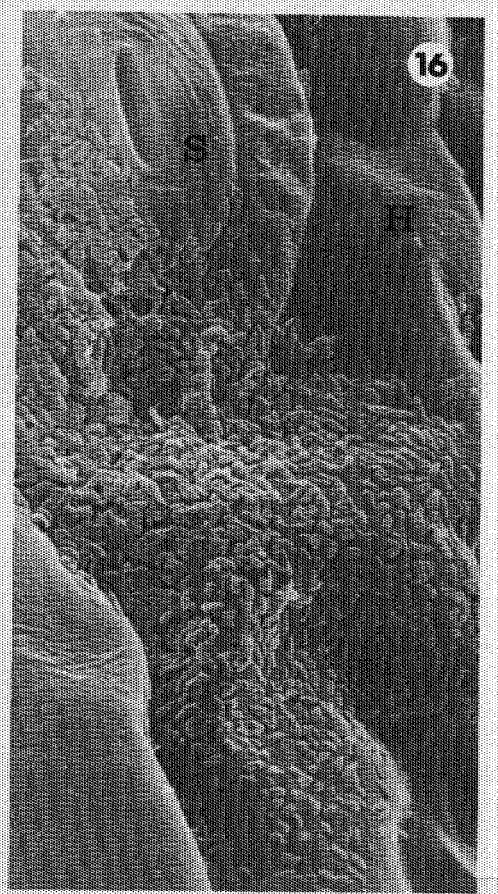
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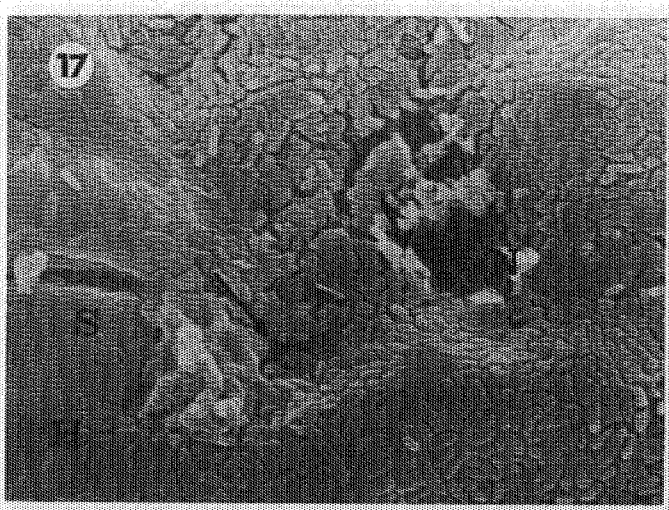
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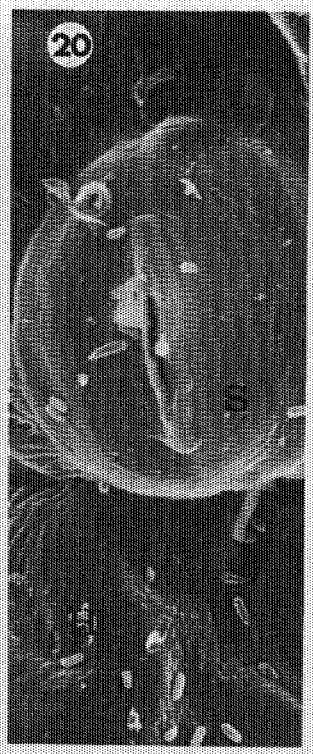
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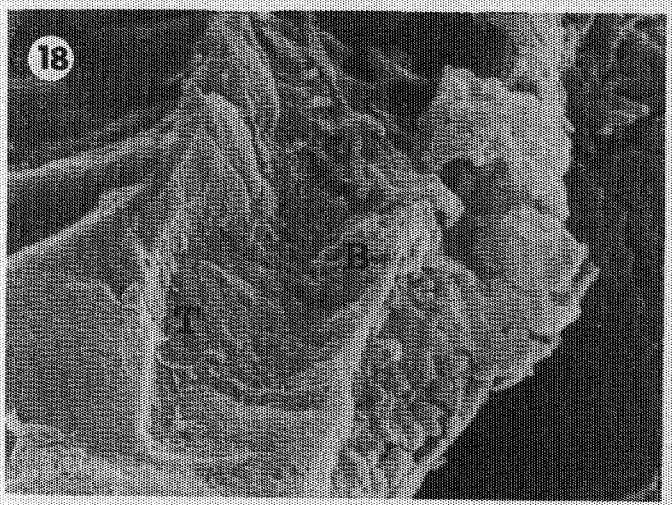
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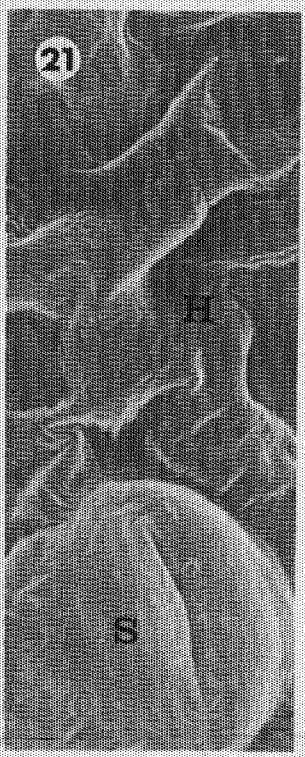
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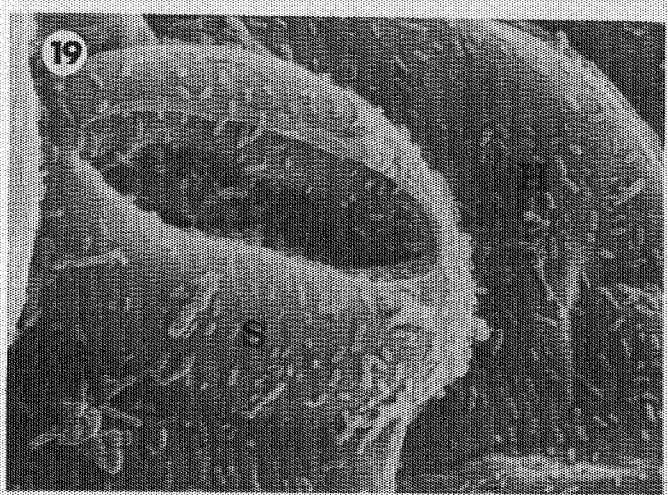
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