

Establishment of an Inactive Population of *Erwinia carotovora* in Healthy Cucumber Fruit

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ABSTRACT

A pathogenic isolate of *Erwinia carotovora* was injected into the centers of cucumber fruits attached to the vine without causing disease. No macroscopic or microscopic symptoms were observed during the 5- to 8- day incubation period. The bacterium, however, was reisolated from the internal tissues of the inoculated, harvested fruits.

Examination of tissue from freshly pectolyzed cucumber fruit revealed that many dead and a few metabolically active cucumber cells contained motile *Erwinia* cells. Bacteria were

microscopically observed in membrane-bound vesicles and free within the cytoplasm. Cucumber cells containing large numbers of bacteria were dead. However, some living cucumber cells, as shown by their ability to plasmolyze and accumulate neutral red into the central vacuole, contained a few motile bacterial rods. The possible significance of *E. carotovora* within living cucumber cells is discussed in relation to the occurrence of soft rot diseases.

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Additional key words: resident bacterial flora, latent infection.

The internal tissues of healthy fruits and vegetables, once generally assumed to be sterile, can contain a diverse bacterial flora (2, 3, 5, 7, 10, 14). Recently we reported (6) on the occurrence of soft rot bacteria within healthy field-grown potato tubers and cucumber fruits, and some factors which possibly activate this potentially pathogenic flora into producing a soft rot. However, our preliminary studies revealed that the percentage of field-grown cucumbers internally infested with these bacteria was extremely variable, ranging from 0-100% in different lots. This variability in natural infestation precluded reproducible experiments on the activation and location of bacteria within the tissue, and thus necessitated the development of a technique that would guarantee almost 100% infestation. Results in establishing a quiescent pathogenic population of *Erwinia carotovora* (L. R. Jones) Holland within healthy, susceptible, cucumber fruits, and their possible location within such tissues are presented in this study. A preliminary investigation has been reported (8).

MATERIALS AND METHODS.—Healthy, undamaged, greenhouse-produced cucumbers (*Cucumis sativus* L., parthenocarpic 'English type') grown within a plastic film greenhouse in sand, were used throughout the study. Fruits, harvested at different stages of maturity were rinsed in running tap water, washed in a detergent solution, and surface-sterilized by bathing in 0.5% NaOCl for 30 minutes. After the surface-sterilized fruit were air-dried under ultraviolet light or filter-sterilized air, standard surface swab techniques showed that the cucumber surfaces were sterile. Cucumbers were aseptically broken open (rather than sliced) under filtered air in a transfer chamber and 50-75 g of tissues removed with a sterile, long-handled scoop. Samples were submerged in 200 ml of sterile distilled water in separate, screw-top, wide-mouthed jars, and incubated at 25 C. Previous experience has shown that obvious growth develops usually within 3 days when bacteria are present. When this occurred, cultures were streaked on nutrient agar (Difco) and sodium polypectate (13) for subsequent

identification. Preliminary studies of over 100 greenhouse-grown cucumbers, collected at various times over a 1-year period, revealed that most were free of internal bacterial contamination. Most importantly, none of these cucumbers was shown to contain soft rot bacteria.

Erwinia carotovora was established in susceptible cucumber tissues by hypodermic injection of 0.5 ml of a standardized cell suspension of the bacterium (1×10^5 to 1×10^6 cells/ml) into the centers of individual young (less than 15 cm in length) to mature (greater than 25 cm in length) fruits attached to the vine. The injection site was approximately equidistant between stem and blossom ends. The standardized suspension was prepared by diluting in sterile water blanks a 24-hour culture of *E. carotovora* grown on glucose-yeast-carbonate slants. This isolate, originally recovered from the internal tissues of a naturally infested cucumber, was capable of macerating cucumber and potato slices within 48 hours. Controls were injected with 0.5 ml of sterile water. All fruits were allowed to mature an additional 5-8 days before harvesting. After detachment and surface sterilization, internal tissue was removed from the immediate area of injection and assayed as described above. In addition, fruits inoculated approximately 5 cm from the stem end were assayed at intervals of 5, 10, 20, and 30 cm from the injection site.

To determine if the population of *E. carotovora* increased or decreased in the tissues of cucumber fruits attached to the vine, four fruits were each inoculated with 0.5 ml suspensions of cells, at concentrations ranging in 10-fold increments from 1.8×10^2 to 1.8×10^7 bacteria/ml. Control cucumbers were injected with sterile water and the experiment was repeated once. Five days after the injections, the fruits were harvested. Following surface-sterilization, 5 g of locular tissue was removed from the area of injection and macerated in a sterile tissue grinder; serial dilutions were plated on nutrient agar. Additionally, four cucumbers, inoculated with 0.5 ml suspension of 1×10^5 bacteria/ml, were picked

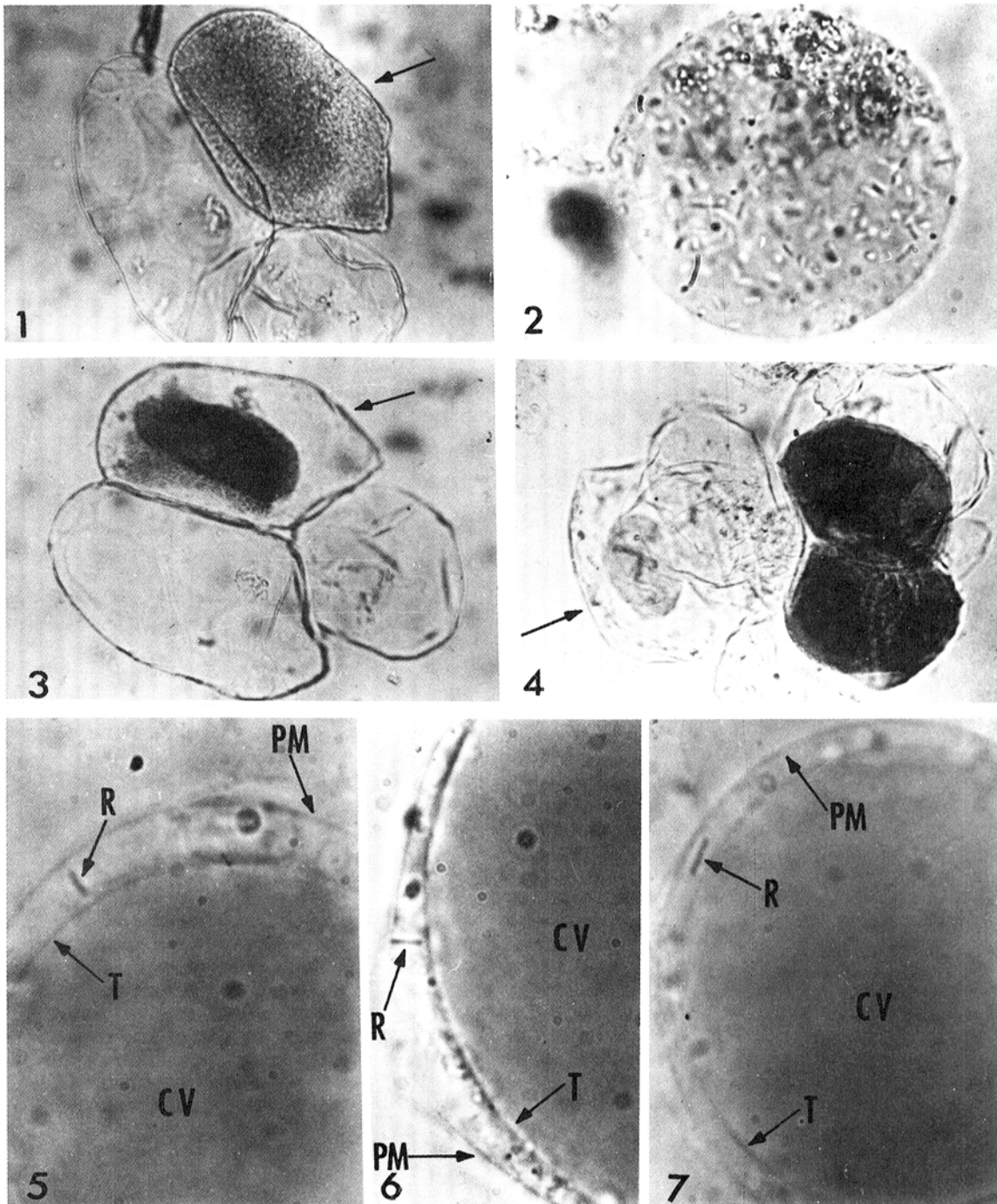


Fig. 1-7. Intracellular localization of *Erwinia carotovora* in living and dead cucumber cells from pectolyzed tissue. 1) Cucumber cell filled with actively motile *E. carotovora* (arrow) ($\times 150$). 2) Bacterial rods of *E. carotovora* entrapped within a plant vesicle occurring in the cytoplasmic debris ($\times 1,020$). 3. Cucumber cell (from Fig. 1) filled with *E. carotovora* and plasmolyzed in a 0.5 M sugar solution (arrow) ($\times 150$). 4) Plasmolyzed cucumber cell containing *E. carotovora* (arrow). The two filled cells at right did not plasmolyze ($\times 150$). 5-7) Bacterial rods of *E. carotovora* (R) in the cytoplasm of plasmolyzed living cucumber cells. The central vacuole (CV) has actively taken up the neutral red dye ($\times 1,020$). PM = plasma membrane; T = tonoplast; CV = central vacuole; R = bacterial rod.

immediately, incubated for 5 hours at room temperature, and then assayed.

RESULTS.—Cucumbers, harvested 5-8 days after

inoculation with the standardized suspension or water only, showed no external or internal, macroscopic or microscopic symptoms. However, *E. carotovora* was

reisolated from 88% of 50 fruits (five lots of 10) inoculated with the standard suspension. *Erwinia* was not recovered from either tissues removed 5, 10, 20, and 30 cm from the inoculation point, or from 30 cucumbers injected with sterile water.

Plate counts of the tissue from four fruits harvested immediately after injection with 0.5 ml of 1×10^5 cells/ml revealed no change in the population of *Erwinia* per gram of tissue. Cucumbers incubated 5 days on the vine had a population increase of approximately 100-fold when inoculated with 0.5 ml of 1.8×10^2 and 1.8×10^3 bacteria/ml, and approximately 10-fold when inoculated with the 1.8×10^4 and 1.8×10^5 concentrations. There was no increase with the 1.8×10^6 concentration. Two of the eight cucumbers inoculated with the 1.8×10^7 suspension rotted on the vine, while the remaining six showed no population increase in the tissue.

Microscopic examination of rotted tissue showed many cucumber cells partially or completely filled with actively motile *Erwinia* (Fig. 1). Bacteria were either free or in membrane-bound vesicles in the cytoplasm, or trapped in vesicles floating freely in cytoplasmic debris of disrupted cucumber cells (Fig. 2). The motile bacterial rods obviously encased in these plant structures were not confused with bacteria adhering to the external surface of plant cells and vesicles, or with plant organelles exhibiting Brownian motion or cytoplasmic streaming. Confirmation that the intracellular bacteria were only *Erwinia* was accomplished by washing individual cucumber cells three to four times with sterile water. During the last washing, the cucumber cell was broken with a glass rod and its contents streaked onto nutrient agar for subsequent identification of the bacteria.

Because many of the cucumber cells that were partially filled with *Erwinia* plasmolyzed in a 0.5 M sorbitol solution (Fig. 3, 4), experiments were undertaken to determine if such cells were alive using a modification of Tribe's neutral red method (15). With this technique, supposedly only living cells accumulate the stain in the vacuoles. This observation was verified using the metabolic inhibitor 2,4-DNP (2,4-dinitrophenol). Approximately 0.5 g of cucumber locular tissue was placed in 20 ml of the pectinase maceration medium of Jensen (4) (pH was adjusted to that of the tissue, 6.5, and the sorbitol to 0.3 M) and incubated for 1 hour at 25 C. Separated cells and small undigested tissue pieces were collected on nylon monofilament screen cloth (30 μ m openings) and gently washed three times with 20-ml aliquots of White's inorganic salt solution (17) with 0.3 M sorbitol at pH 6.5. Cells and tissue segments were resuspended in 20 ml sorbitol-salt solution containing 1×10^{-4} M 2,4-DNP. After cultures were incubated under light of 4,304 lx (400 ft-c) at 25 C for 2 hours, 0.1 ml of 1.0% neutral red solution was added. Observations from 30 minutes to 18 hours revealed that 2,4-DNP prevented active accumulation of neutral red into the vacuoles, though protoplasts remained intact. Cells incubated in the absence of 2,4-DNP readily took up the dye into their vacuoles, supporting the premise that they were alive and metabolically active.

Application of the neutral red technique to tissue removed from the advancing margin of freshly pectolyzed cucumber tissue (less than 24 hours old) revealed that almost all cells containing bacteria were dead. However,

close microscopic examination showed that some cucumber cells, which accumulated neutral red into the central vacuole, also contained a few bacterial rods. These bacteria were observed swimming throughout the cytoplasm, bouncing off the inside of the plasma membrane, or entrapped in membrane-bound vesicles within the cytoplasm, but never penetrating the tonoplast into the vacuole (Fig. 5, 6, 7).

DISCUSSION.—Two facts, (i) the reisolation of *E. carotovora* from symptomless cucumbers 5-8 days after inoculation and (ii) the recovery of soft rot bacteria from apparently healthy, naturally infested, plant tissue (6), suggest that these pathogenic bacteria may exist in a commensalistic or quiescent state. An explanation of their inactivity in both naturally and artificially infested susceptible plant tissues is still conjectural. Possibly the bacteria are limited in growth because of inhibitory substances present in the tissue; pectic enzyme inhibitors may suppress the elaboration of the bacterial enzymes required for pathogenesis. Activation of the bacteria may result if the physiological condition of the fruit is disturbed from lack of oxygen, unbalanced nutrition, temperature stresses during storage, or a mechanical-enzymatic injury inflicted by a pathogenic fungus (6). This may explain the occurrence of many storage and field rots previously attributed to external bacterial contamination.

Apparently, resistance also can be broken when high populations of bacteria are introduced into tissues. Soft rot of cucumbers attached to the vine occurred frequently when populations greater than 1×10^7 bacteria/ml were injected into the fruit. In preliminary studies, we repeatedly observed that 25-30% of the cucumbers inoculated with such populations usually rotted on the vine within 3 days, while the remainder showed no symptoms after the 5-8 day incubation period.

The ability of many cucumber cells, which were partially filled with *Erwinia*, to plasmolyze, indicated that the plasma membrane was still intact and able to function as a semipermeable membrane. The discovery that some cucumber cells containing *Erwinia* were alive merits further attention. How these bacteria are able to enter the plant cell without killing it, is unknown. Jones and Paton (5) postulate that invasion of potato cells is by L-phase elements of *E. carotovora*, which later revert to normal, actively multiplying bacterial rods. However, they did not observe L-phase bacteria in cells from young macerated potato tissues, but only in cells of 30- to 50-day-old pectolyzed potato tissues. Most likely, such tissues had long been dead. On the other hand, we have seen bacterial rods present in the cytoplasm of living cucumber cells taken from freshly pectolyzed tissue; i.e., less than 24 hours old.

These bacteria can, perhaps, successfully penetrate the wall and membrane without disturbing the structure or physiology of the cell. Scott (11) illustrated that some pits in parenchyma cells of *Ricinus communis* can measure 1 μ m or greater in diameter. If such pits in cucumber cells approach this size, entry by *Erwinia* is possible. Once penetration of the primary wall has been achieved, uptake could occur by endocytosis (16). Davey and Cocking (1) demonstrated by means of electron micrographs that pea leaf protoplasts engulf *Rhizobium* cells into membrane bound vesicles in the cytoplasm during experimental

digestion of the surrounding cell wall. They also observed bacteria enclosed in vesicles in the cytoplasmic debris from burst protoplasts. Our results show that soft rot *Erwinia* also exist free or in membrane-bound vesicles within the cytoplasm of living cells taken from freshly pectolyzed tissue. Experimental proof that bacteria occur intracellularly in a quiescent state is required before conclusions can be made about their intracellular occurrence (9, 12) and significance in healthy, naturally infested, plant tissue.

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