Cytology and Histology

Colonization and Penetration of Intact Canola Seedling Roots by an Opportunistic Fluorescent *Pseudomonas* sp. and the Response of Host Tissue

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The authors wish to express their gratitude to G. Braybrook, Department of Entomology, University of Alberta, for expert assistance with the scanning electron microscope studies and to Linnea Sorlie for technical assistance.

Financial support from the Natural Sciences and Engineering Research Council of Canada and from the Central Research Fund, University of Alberta, is gratefully acknowledged.

Accepted for publication 24 February 1987 (submitted for electronic processing).

ABSTRACT

Campbell, J. N., Cass, D. D., and Peteya, D. J. 1987. Colonization and penetration of intact canola seedling roots by an opportunistic fluorescent *Pseudomonas* sp. and the response of host tissue. Phytopathology 77:1166-1173.

A fluorescent pseudomonad, isolated from canola (rapeseed) plant material in the field and designated *Pseudomonas* rp2, has been shown to be an opportunistic pathogen for immature canola roots, causing inhibition of growth and development. Disinfested seeds were inoculated with *P.* rp2 and allowed to germinate under axenic conditions in vitro. The sequential events of surface colonization, invasion, and lesion formation in the emerging intact root were monitored. Intact seedling roots of *Brassica campestris* 'Candle' (canola) have been shown to be susceptible to infection by *P.* rp2 for a period of 4 to 5 days after germination. Roots infected during this period exhibit surface microcolonies, and penetration of root tissue appears to be associated with such colonies, occurring by bacterial intrusion along epidermal cell anticlinal walls. Initial and subsequent wall penetration appears to be accompanied by loosening of cellulose microfibrils, possibly facilitated by elaboration of a cell-associated bacterial pectinase. Bacterial penetration occurs throughout the root cortex, terminating in the formation of large colonies in the intercellular spaces. No bacterial penetration through the endodermis nor to any tissue layer interior to the endodermis has been observed. Some bacterial cells in cortical intercellular colonies exhibit cytoplasmic inclusions not seen in in vitro grown cells, which could result from the unique nutritional environment in vivo. Although no intracellular bacteria have been observed, evidence of overall host tissue distress has been obtained in the form of altered host cell ultrastructure and an altered fluorescein diacetate reaction in those regions of roots colonized by the bacteria.

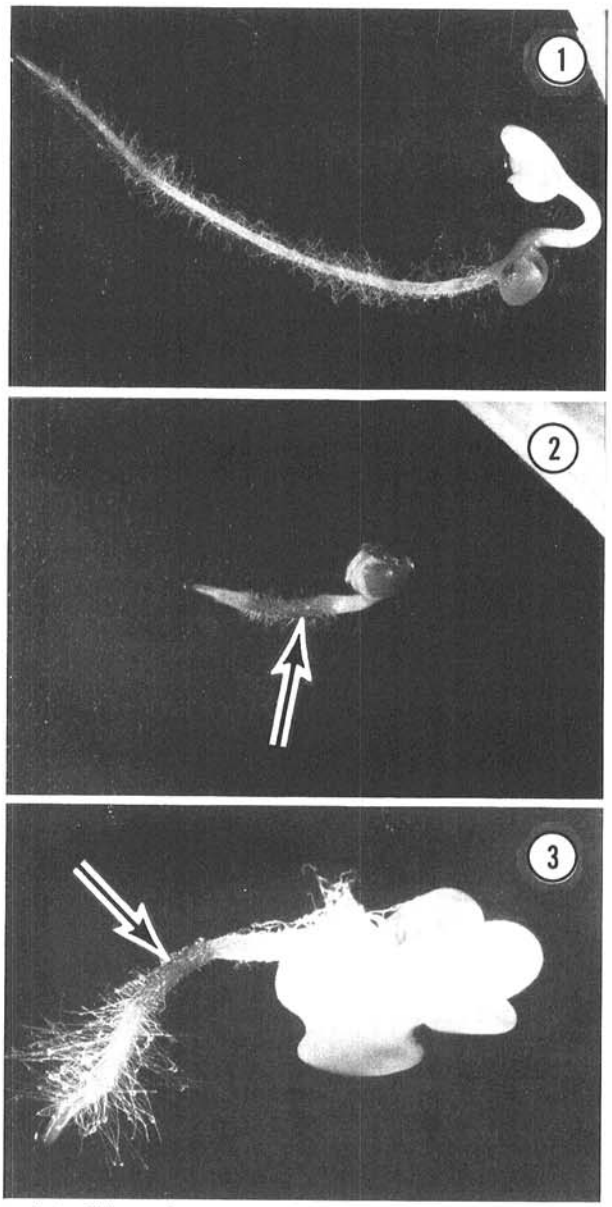
Among members of the genus *Pseudomonas* there are numerous species that are pathogenic to plants. In some, the pathogenic principles are well established, for example syringomycin production by *P. syringae* pv. *syringae* (14), whereas in others, which Suslow referred to as minor pathogens (15), the pathogenic principles are more subtle and less well understood. For example, nutritional competition by production of chelating or scavenging agents such as siderophores (16), alteration of root cell permeability (1), and production of mild toxins and hormone-like compounds that affect a variety of symptoms (reviewed by Suslow [15]) have been implicated. Even less well understood are the opportunistic pathogens, those species that are normally

innocuous residents of the environment but that can initiate disease in the host if, and only if, specific conditions in the host, the microorganism, and the environment are met.

In 1986, Campbell et al (2) reported the isolation of a species of fluorescent pseudomonad capable of causing an inhibition of root development in sprouting *Brassica campestris* L. 'Candle' (canola) under laboratory and field conditions. The present report deals with the sequence of events on intact developing roots from initial exposure to the bacteria on root emergence through to final colonization of the host tissue.

MATERIALS AND METHODS

Microorganisms and culture conditions. *Pseudomonas* rp2 is a fluorescent pseudomonad isolated from field-grown canola in northern Alberta and is an opportunistic pathogen for immature canola roots (2). Other species were obtained from the culture collection of the Department of Microbiology, University of Alberta. For inoculation of seeds, bacteria were grown in trypticase soy (ts) broth (Baltimore Biological Laboratory, Baltimore MD) for 18 hr at 30 C with shaking. Stock cultures were maintained on ts broth plus 1.5% agar.



Figs. 1–3. Effect of presence of *Pseudomonas* rp2 on early root development in canola seeds sprouting in vitro. **1,** Uninoculated seedling, 36 hr postplanting showing normal development (×3.2). **2,** Seedling, 36 hr postplanting from a seed inoculated with *P.* rp2. This root damage was irreversible and such plants did not survive. **3,** Seedling, 96 hr postplanting from a *P.* rp2-inoculated seed. In such cases, root growth and development were delayed, but the plants ultimately recovered. Arrow indicates lesion (×5.8).

Growth and inoculation of plant material. *B. campestris* 'Candle' (canola) seeds were obtained from the Department of Plant Science, University of Alberta.

For experiments involving single measurements or disruptive analyses, a maximum of 10 disinfested (control) or disinfested and inoculated (infected) seeds were distributed on filter paper in a sterile plastic petri plate. The paper was kept saturated with sterile distilled water, and aseptic procedures were employed throughout. The plates were incubated at 18 C in the dark.

Dry canola seeds were disinfested by immersion in 0.05% NaOCl with gentle shaking for 10 min, rinsed in sterile distilled water, and inoculated, if appropriate, by immersion for 1 min into an aqueous suspension of 10^6 colony-forming units (cfu) per milliliter of washed, late log phase cells, grown in ts broth, drained, and dried as previously described (2).

Electron microscopy. For transmission electron microscopy (TEM), samples were fixed for 18 hr in glutaraldehyde (4%, w/v) plus acrolein (5%, w/v) in 0.1% cacodylate buffer, pH 6.8, dehydrated in a graded ethanol series, and embedded in either EPON 812 or Spurr's resin. Thin sections were cut using a Reichert OM U2 ultramicrotome, mounted, stained according to the uranyl acetate:lead citrate procedure of Reynolds (13), and examined in a Philips EM-300 at 60 kV.

For scanning electron microscopy (SEM), samples were fixed in glutaraldehyde and acrolein as described above, subjected to critical point drying, and examined in a Cambridge 250 Stereoscan microscope. In some cases, tissue samples were prepared for SEM by the following modification of the resin removal procedure of Hogan and Smith (7). Spurr-embedded, 2.5-μm sections of root tissue were treated by the following sequence: 0.5% methanolic KOH:benzene:acetone (1:1:1), 20 min; 1% (v/v) acetic acid in methanol, 1 min; 100% methanol, 5 min; 0.3% H_2O_2 in methanol, 45 min; 100% methanol, 5 min; amyl acetate.

Light microscopy. For light microscopy, 1.5-μm sections were mounted on autoradiography slides and stained with toluidine blue or Sudan Black B (12). Whole roots were treated using the Heslop-Harrison fluorescein diacetate procedure, as modified by Widholm (6,17). Samples were examined in a Zeiss Photomicroscope I equipped with an epifluorescence illuminator and a model M-63 camera.

RESULTS

Alteration of root development induced by *P.* rp2. When disinfested canola seed is inoculated with *P.* rp2 and allowed to germinate in vitro, inhibition of root growth occurs, which is accompanied by the eventual appearance of a visible lesion on the emerging root (2). This lesion persists even in those plants that recover. The contrast in the gross appearance between normal roots from uninoculated seeds (Fig. 1) and inhibited roots from *P.* rp2-inoculated seeds (Figs. 2 and 3) is shown.

Sequence of events in infection of emergent root by seedborne *P.* rp2. *Colonization of root surface.* Seeds were inoculated and allowed to sprout as described. At various times before development of the visible lesion, root tissue was excised, prepared appropriately, and examined as an intact surface in the SEM or as thin sections for light microscopy or TEM. Root surfaces from uninoculated seed showed no detectable surface bacteria (not shown), whereas those from *P.* rp2-inoculated seed bore a large surface population with bacteria in, and intruding deeply into, surface furrows (Figs. 4 and 5). There was a fibrillar material observed associated with bacteria adherent to the plant tissue (Figs. 5–7). Its nature and potential role in adherence are discussed later. Bacterial microcolonies were observed at what appeared to be preferred sites for bacterial replication (Fig. 8).

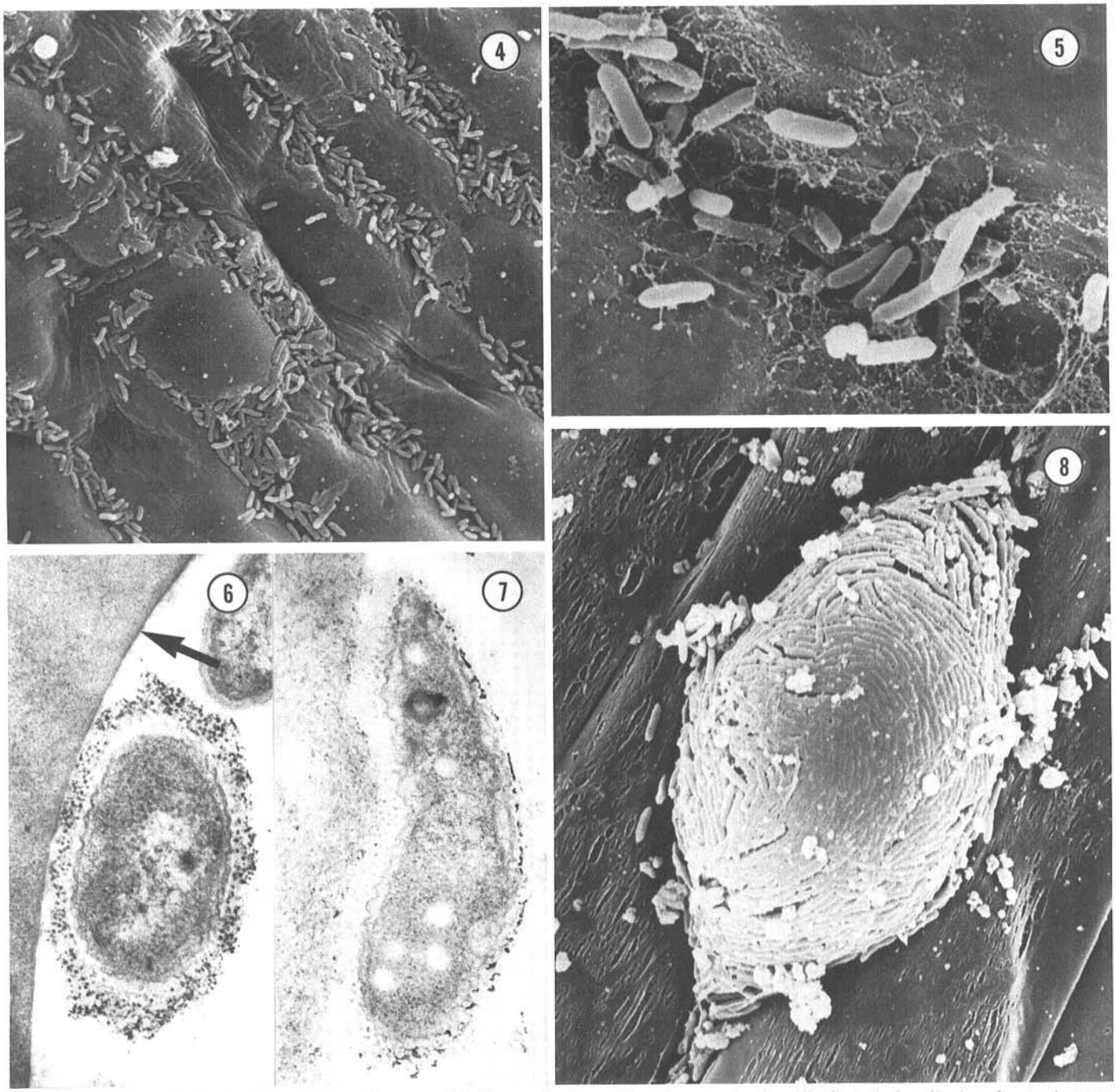
Penetration of intact root tissue. Bacterial cells were often located on the root surface adjacent to anticlinal wall junctions between epidermal cells. In such locations, they could be observed

on the intact cuticle, in which case no evidence of degradation of the cuticle by the bacteria was seen (Fig. 6) or under cuticular flaps (Fig. 9). When in direct contact with epidermal cells, the bacteria appeared to disrupt the integrity of the superficial anticlinal wall junction (Figs. 10 and 23) with the result that the cells were pushed apart and progression of the bacteria into the root tissue proceeded via this intercellular route (Fig. 11). The persistence of the microfibrils as the cells were pried apart is noted. Further penetration of the bacteria into cortical intercellular spaces occurred with accompanying degradation of the middle lamellar region (Figs. 12 and 13).

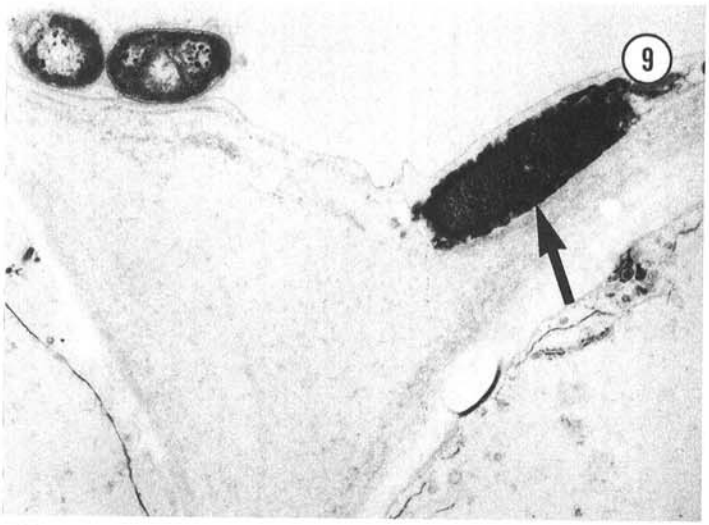
Internal colonization of root cortex. The bacteria not only penetrated the intact root via the intercellular spaces but were able to replicate in this environment (Fig. 14). Progressively later stages in the infection and further colonization of the middle lamella region of the cortex by the bacteria are seen in Figures 15–18.

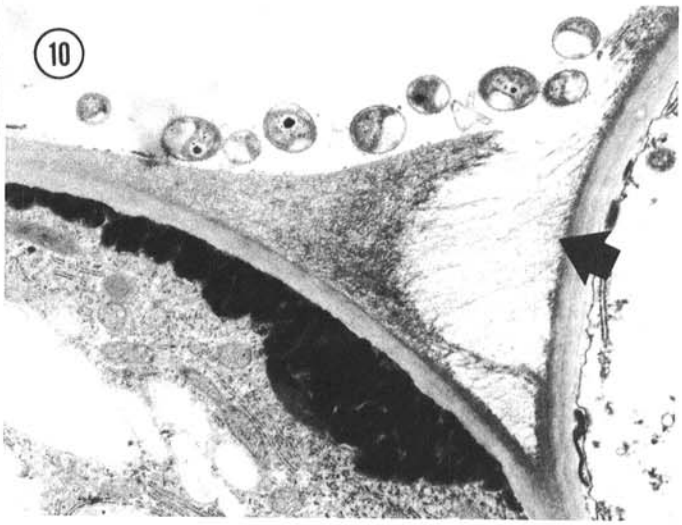
Distribution and extent of inward invasion of root tissue by P. *rp2.* The gross pathology of this infection showed the presence of discrete lesions with no evidence of systemic spread of the pathogen (Figs. 2 and 3). To establish the boundaries of this colonization, thin sections of the various tissue sites were examined microscopically for the presence of bacteria. As has been shown, there was extensive and progressive colonization of the epidermal and cortical regions. This inward progress was halted at the Casparian strip on the radial walls of the endodermis (Figs. 19 and 20). Membrane regularity, wall homogeneity, absence of a distinguishable middle lamella, and plasmolysis at the edge of the strip are all characteristic of the endodermis. The normal cytology of the endodermis and of the interior vascular region indicates that these regions, unlike the cortex, are unperturbed by the adjacent bacterial colonization (Fig. 21).

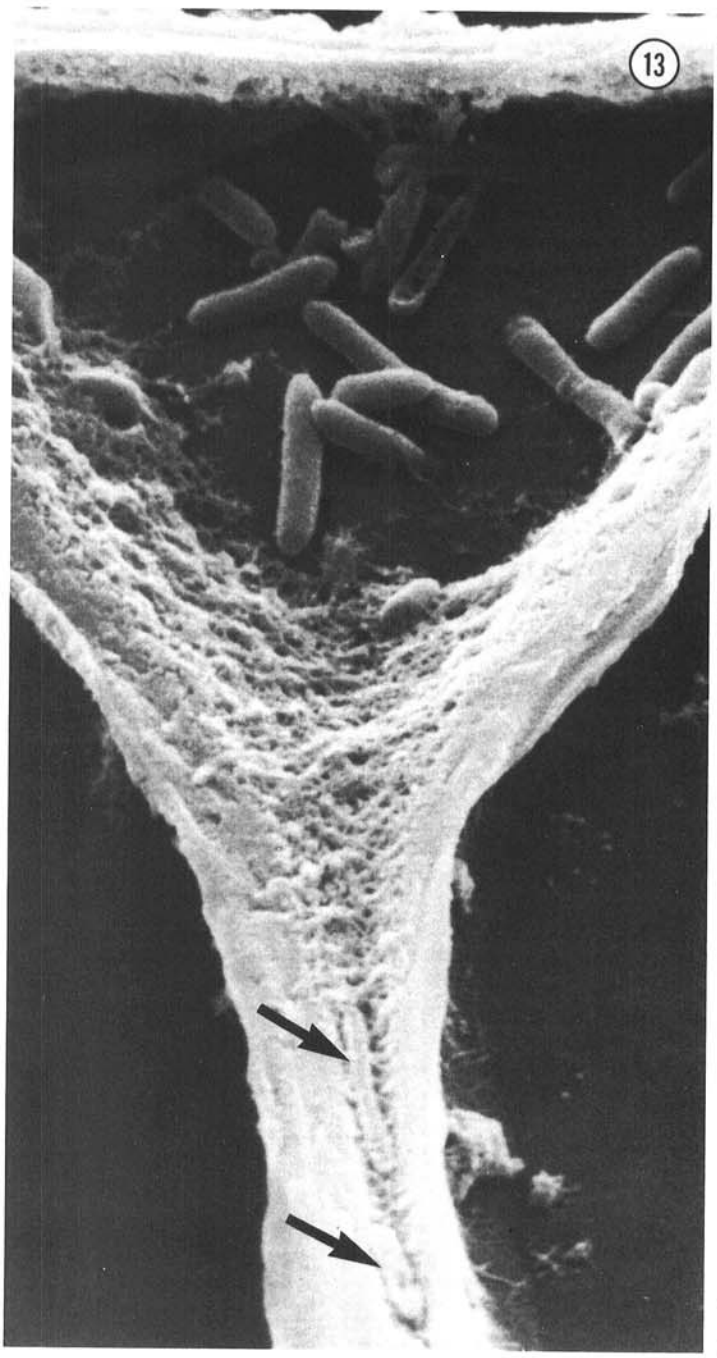
Host response to infection. There is evidence of host tissue

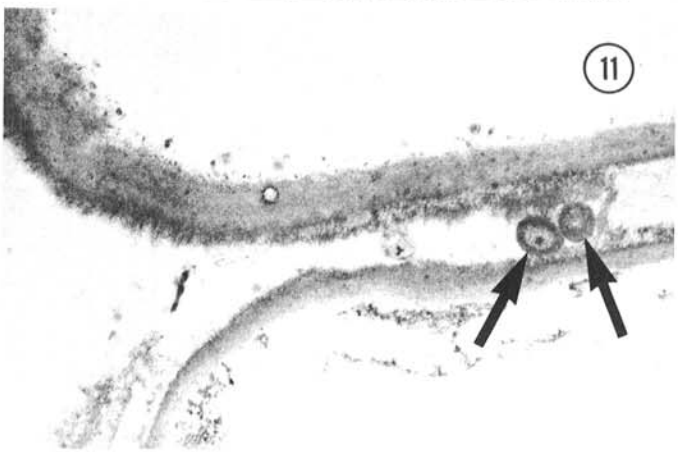


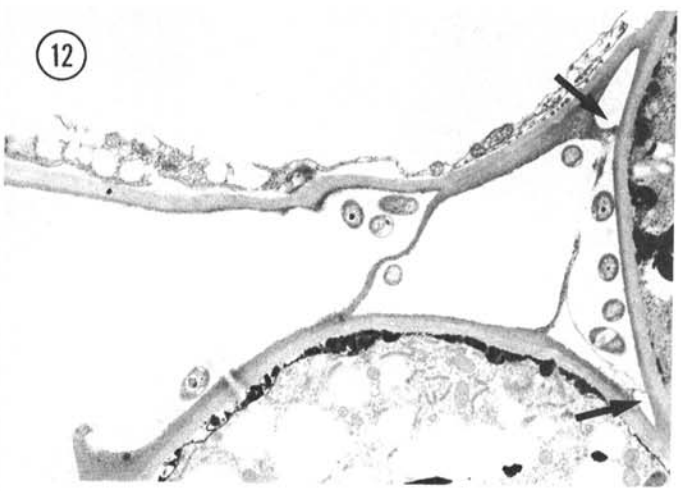
Figs. 4–8. Colonization of surface of root by *Pseudomonas* rp2, 24 hr postplanting. **4,** Bacteria on root surface, distributed primarily along furrows between epidermal cells (×1,180). **5,** Bacterial penetration deeper into a furrow. The presence of fibrillar material around the bacterial cells is noted (×5,250). **6,** Bacterium in direct contact with root cuticle. Arrow indicates waxy layer (×44,800). **7,** Bacterium in intimate contact with root cuticle. The waxy layer appears to be absent (×44,800). **8,** Microcolony of bacteria on root surface (×2,000).







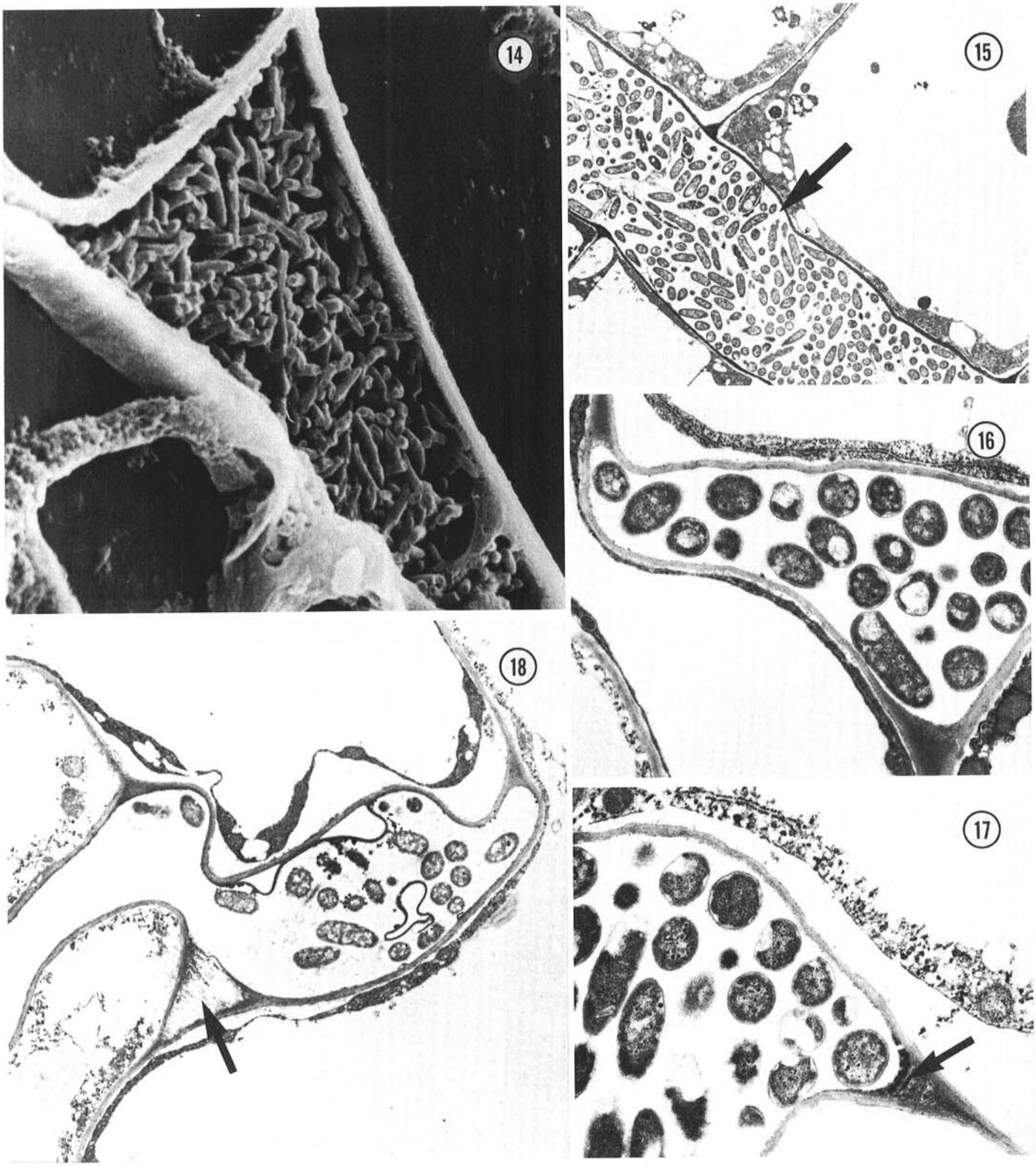




Figs. 9–13. Penetration of root tissue by *Pseudomonas* rp2, 48 hr postplanting. **9,** Bacterial cell on the root surface under a flap of cuticular wax (arrow) (×15,200). **10,** Bacteria-associated degradation of anticlinal wall junction in root epidermis. Arrow indicates wall lesion (×12,000). **11,** Further penetration of bacteria into an anticlinal wall junction. Root surface is to the left (×15,200). **12,** Bacteria just before entry into cortical intercellular space. Root surface is to the left (×5,200). **13,** Section of root cortex from which resin has been removed showing bacteria in an intercellular space (above), and penetrating along middle lamella (arrows) (×9,800).

damage other than that directly attributable to bacterial invasion along middle lamellae. Under growth-permitting conditions, infection resulted in inhibition of root growth and delay of maturation in whole plants (2). Both epidermal and cortical cells of the host root exhibited osmiophilic inclusions, uniquely adjacent to the sites of bacterial colonization and bacteria-induced wall degradation (Figs. 10, 12, 22, and 23). In whole root mounts treated with fluorescein diacetate, tissue in the region of the lesion failed to convert the dye to its fluorescent form, indicating reduced viability, whereas regions proximal to the lesion, as well as uninfected roots, exhibited normal activity (Figs. 24 and 25).

The bacteria colonizing the intercellular spaces frequently



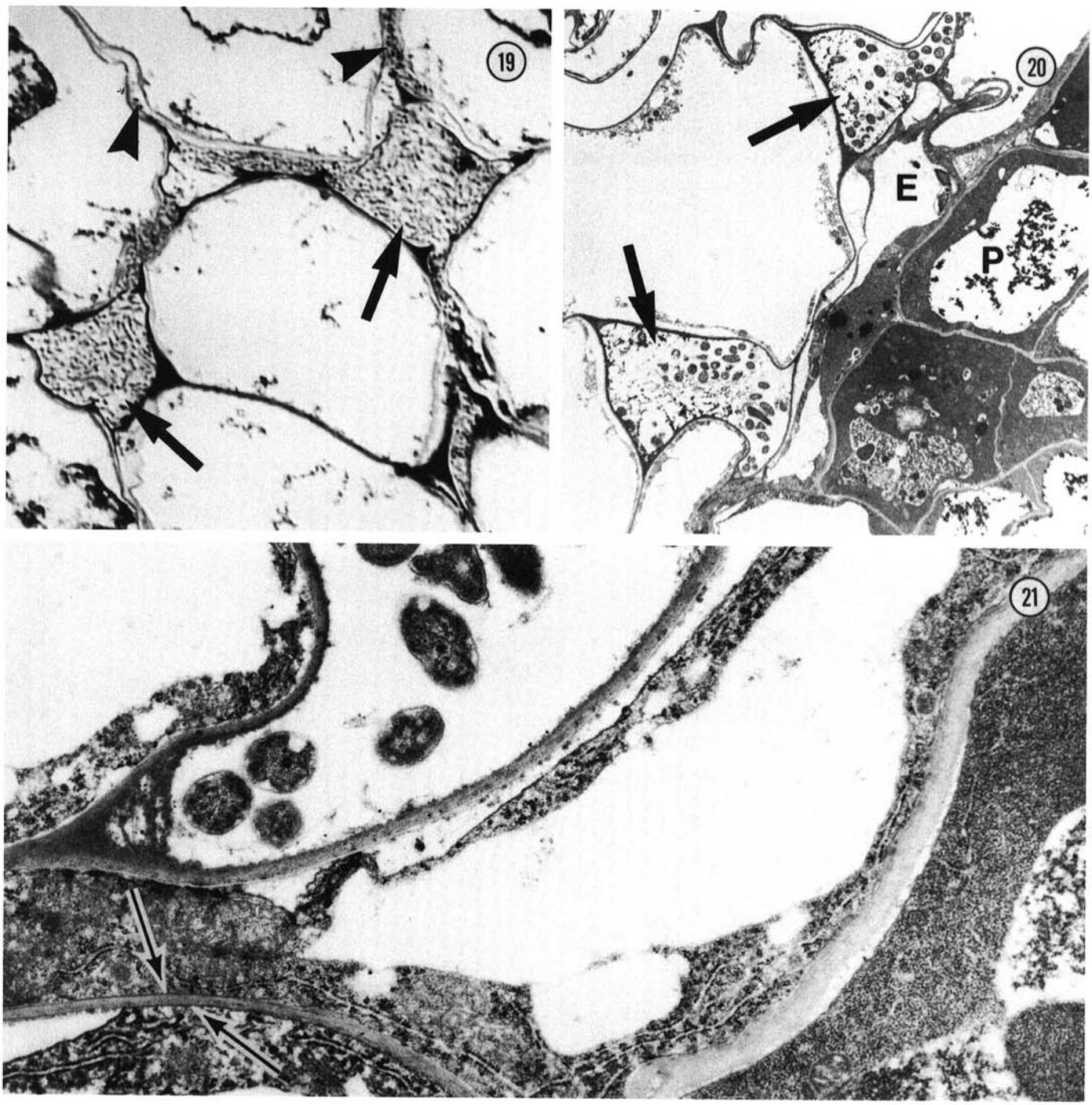
Figs. 14–18. Internal colonization of root tissue by *Pseudomonas* rp2, approximately 60 hr postplanting. **14,** Transverse section of root cortex after resin removal procedure showing colonization of intercellular space (×3,300). **15,** Bacteria colonizing cortical intercellular space (arrow). No apparent damage to adjacent cortical cells has yet occurred (×44,800). **16,** Further colonization of cortical intercellular space by bacteria. There is no apparent damage to wall junctions remote from colonization site (×15,200). **17,** Early stage of damage (arrow) to cortical cell wall adjacent to the colonization site (×19,000). **18,** More extensive damage (arrow) to wall adjacent to a bacterial colony (×7,000).

exhibited what appeared to be cytoplasmic inclusions that were not evident in in vitro grown cells under our conditions (Figs. 10–12).

DISCUSSION

This report presents results of a histological study of the colonization and penetration of intact canola seedling roots by a seedborne population of the fluorescent pseudomonad, *P.* rp2. The object is to elucidate details of an opportunistic infection that is of economic significance in field-grown canola, as well as to develop a model system for further investigations of infections of this type.

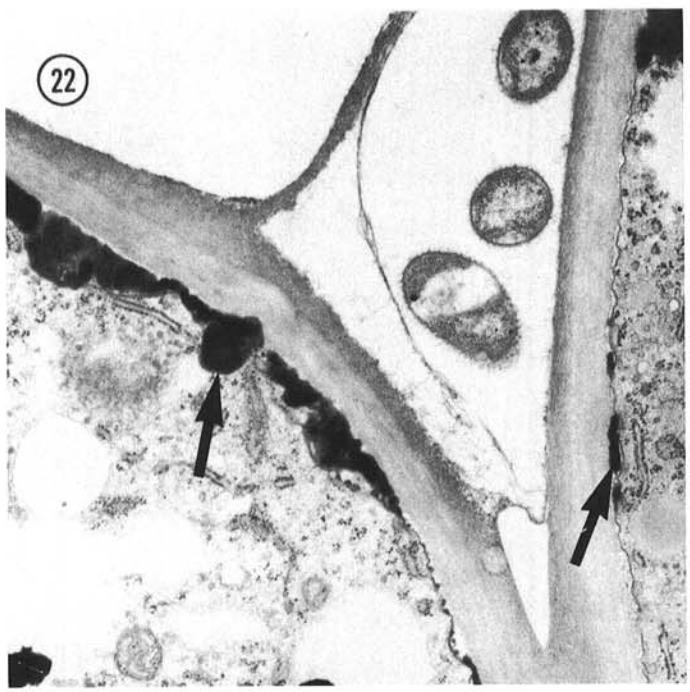
P. rp2 readily survives and replicates on intact canola roots. This is not, in itself, significant, because the rhizoplane is a preferred environment to a wide variety of commensal and neutralistic, as well as pathogenic species of bacteria. However, if colonization by *P.* rp2 occurs within a short time after emergence, this normally innocuous pseudomonad produces disease in the host (2), and, in this situation, colonization of the root surface is an essential first step to subsequent penetration and host damage. Simple contact between bacteria and cuticle does not necessarily lead to invasion, and we have been unable to find direct evidence either of degradation of underlying cuticle by adherent *P.* rp2 (Fig. 6), or of production of cuticle-degrading enzymes by *P.* rp2 in vitro. If, however, the bacterial cell comes in contact with the underlying epidermal cells, perhaps through a break in the cuticle, root penetration can follow. In this system, we have no evidence for a lectin-mediated, site-specific attachment mechanism as described by Dazzo et al (4). The fibrillar material seen associated with the

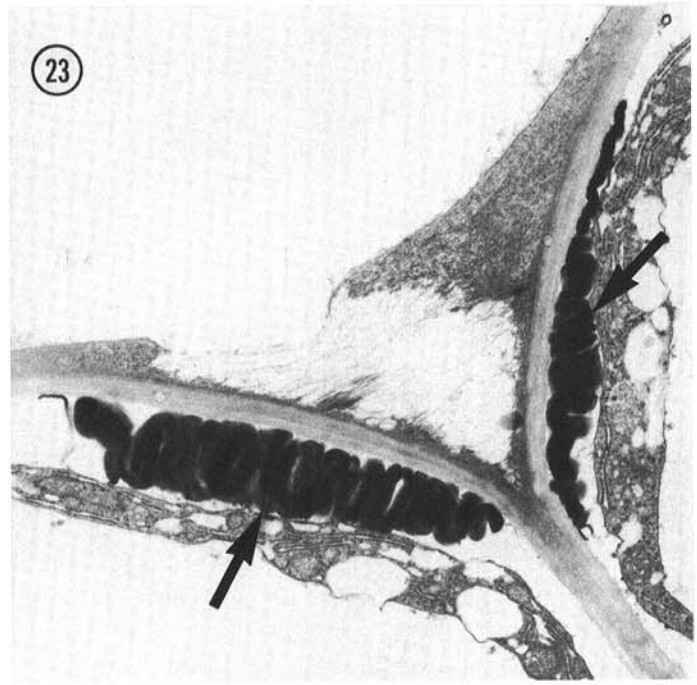


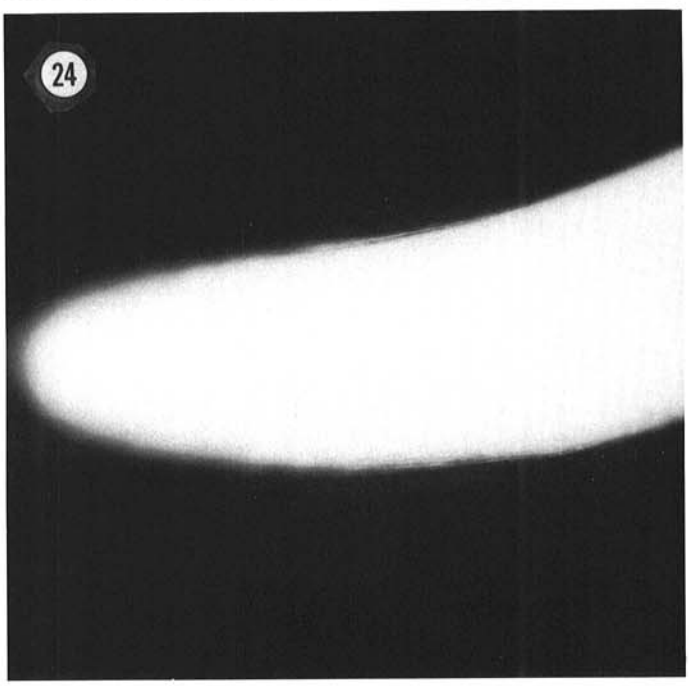
Figs. 19–21. Distribution and limitation of penetration of *Pseudomonas* rp2 in root cortex, 72 hr postplanting. **19,** Nomarski micrograph of 1.5 μm tissue section showing bacteria in intercellular spaces (arrows) and penetrating along middle lamellae (arrows) (×985). **20,** Root cortex showing inner limit of bacterial invasion at the root endodermis (E). Note the bacterial colonies (arrows) and the pericycle (P) (×2,800). **21,** Higher magnification of the lower region in Figure 20 showing a typical, intact Casparian strip (arrows) (×15,200).

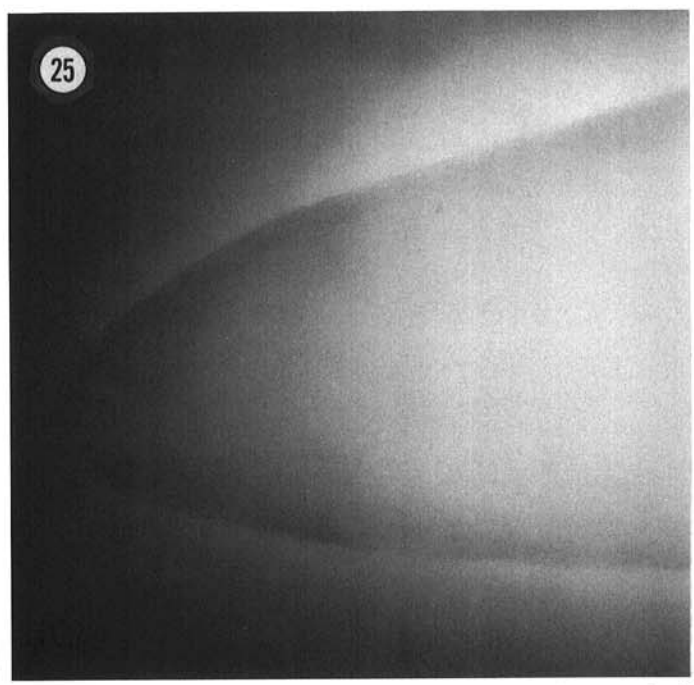
bacteria (Figs. 5 and 6) is probably the mannuronic acid-containing extracellular polysaccharide capsule produced by *P.* rp2 (2, *unpublished data*), and this structure may be involved in adherence. The formation of microcolonies at wall junctions (Fig. 8) suggests that these represent preferred sites of replication and subsequent invasion after the initial contact has occurred. Whether this contact is random or whether there is some positive chemotactic attraction to these preferred sites, as described for species of *Rhizobium* (5), has not been established. These microcolonies are relatively common on an infected root, but they are easily disrupted and can only be detected early in the infection before they become overgrown. These preferred sites of replication represent logical points for subsequent penetration, which is exclusively via anticlinal wall junctions between epidermal cells (Figs. 10–12,23). These correspond in appearance to the pectin-rich cuticular pegs described by Holloway (8).

As the middle lamella is progressively disrupted and the cells separate, a microfibrillar component of the wall or lamella becomes evident (Figs. 10 and 13). The observation that *P.* rp2 in vitro produces a cell-associated polygalacturonic acid hydrolase but no detectable cellulase activity (2, *unpublished data*) raises the possibility that the mechanism of intercellular invasion involves the degradation of the pectin or pectin-related component(s) of the middle lamella by the bacterial enzyme, with the subsequent disruption of tissue integrity. Purification and characterization of this enzyme, as well as its activity on host plant tissue, are subjects of a separate study. The penetration of the epidermal layer gives the bacteria access to the intercellular space system of the root









Figs. 22–25. Host response resulting from infection of the root with *Pseudomonas* rp2. **22,** Osmiophilic deposits (arrows) between plasmalemma and cell wall in host cells adjacent to site of bacterial colonization, 72 hr postplanting. To the left is an epidermal cell and to the right an outer cortical cell (×18,400). **23,** Similar osmiophilic deposits (arrows) in host epidermal cells adjacent to bacterial penetration site (×16,100). **24,** Fluorescence micrograph of uninfected root stained with fluorescein diacetate, 96 hr postplanting (×125). **25,** Fluorescence micrograph of an infected root stained with fluorescein diacetate, 96 hr postplanting. The absence of fluorescence is noted (×125).

cortex (Figs. 12–19). The pattern of bacterial degradation of the pectin wall component is repeated, this time involving the middle lamella rather than the cuticular peg of the epidermal cells. The bacteria colonize both the normal intercellular spaces (Fig. 15) as well as those areas loosened and separated by bacterial action. In the bacterial degradation of complexed biopolymers, such as pectin in plant cell walls, it might be expected that exocellular hydrolases, such as are produced by *Erwinia* (3), would be more efficient than a cell-associated enzyme such as is present in *P.* rp2 (2). This is not always the case. The cell-associated chondroitin sulfate degrading enzyme(s) of *Bacteroides thetaiotaomicron* has been shown to digest chondroitin sulfate, which is complexed with proteoglycan, nearly as efficiently as it does free substrate (10). This cell-associated activity of *Bacteroides*, many species of which are also opportunistic pathogens, does not contribute to invasiveness, but it enables the bacteria to use a substrate that exists in a complexed form in the in vivo environment, in this case the mammalian colon.

Inward penetration of the pseudomonad seems to be stopped at the endodermal layer (Figs. 20 and 21). Bacterial cells have never been observed in the vascular system, and the endodermis, with its suberized Casparian strip, appears normal, even when the adjacent cortex is infected (Fig. 21). This region, which is a known barrier to apoplastic water transport in roots (11), may also represent the barrier to bacterial progress. This is in accord with the absence of systemic symptomatology in this disease.

Although the pectinase may be important to invasion by the bacteria, it obviously is not the sole pathogenic factor involved. Host cells are not only separated, they exhibit necrosis, and the overall growth of the root is interrupted (2). This is indicated by the appearance of osmiophilic deposits between the plasmalemma and the wall of host cells adjacent to bacterial colonies. Increased osmiophilia has been observed in a number of other degenerating plant cell systems (9). Also, there is a dramatic difference between normal and infected tissue in response to fluorescein diacetate staining (Figs. 24 and 25). The latter tissue obviously has lost considerable fluorescein diacetate esterase activity, indicating extensive loss of viability. To date it has not been possible to demonstrate the production of phytotoxins or of plant tissue-degrading enzymes other than polygalacturonic acid hydrolase in *P.* rp2 in vitro. However, the observation that bacteria colonizing intercellular spaces characteristically exhibit electron-dense inclusions not seen in cells grown in vitro (Figs. 16 and 17) suggests that this intercellular environment is unique and could possibly elicit synthesis, by the bacteria of phytotoxic compounds not produced in artificial media. Further study of this aspect centers on bacteria grown in plant tissue and dispersed cell culture.

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