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A SCANNING ELECTRON MICROSCOPE STUDY OF THE HOST-PATHOGEN INTERACTION
OF PHYTOPHTHORA INFESTANS WITH POTATO TISSUE

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ABSTRACT

The fungus *Phytophthora infestans* is the causative agent of late blight in potatoes. Certain varieties of potato exhibit vigorous resistance to infection by this pathogen. This study demonstrates structural aspects of the growth and development of *Phytophthora infestans* on both susceptible and resistant tubers.

Slices of varieties Kennebec (resistant) and Houma (susceptible), inoculated with *P. infestans*, race 4, were sampled during germination, penetration, and infection stages of fungal growth by removing cores with a sterile cork borer. Suitably trimmed tissue (6 mm x 4 mm) was fixed in fresh 2% glutaraldehyde (0.15 M phosphate, pH 6.0) for 24 hours, serially exchanged into ethanol and then into amyl acetate before critical point drying using CO₂.

Early stages of fungal growth were studied by inoculating thin sections (200 μ) of potato tissue with spore suspension. Sections were monitored by light microscopy during incubation in order to identify germ structures prior to preparation of the specimen for SEM. The sections, on a microscope slide, were frozen in Freon 22 and freeze dried. When dry, the specimens retained enough rigidity for easy trimming.

The resistance of Kennebec was exhibited by inhibition of mycelial growth. The inoculated surface of the susceptible Houma developed an abundant mycelium which completely covered the surface and extended for 1 or 2 cells into the tissue. The demarcation between infected cells and healthy cells was marked.

KEY WORDS: Scanning Electron Microscope, Potato, Fungus, *Phytophthora infestans*, Disease Resistance, Plant Pathology

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Introduction

The scanning electron microscope (SEM) has been shown to be useful in elucidating fungal morphology (1,2,3,4) and the topography of internal structures of plants (5). Recently, an investigation at this laboratory into the chemical basis of the hypersensitive resistance exhibited by certain varieties of potatoes when infected by incompatible races of Phytophthora infestans has prompted a microscopic study of structural aspects of the infective process. Phytophthora infestans produces the disease known as late blight which, in epidemic occurrences, has been responsible historically for extensive economic losses in the production of potatoes.

In an incompatible reaction, penetration of the living potato cell by the fungus elicits a hypersensitive resistant response which involves localized accumulation of phenolic compounds (lignin, chlorogenic acid) and terpenoids (rishitin, phytuberin) and altered patterns of hyphal penetration and growth.**Host cell death (cessation of protoplasmic streaming) occurs rapidly (30-60 min)(6) in a restricted area. Necrotic areas appear as small darkened areas in the tissue, and fungal growth is effectively contained.

In the susceptible or compatible reaction, the resistant response, including the production of certain response compounds, is suppressed.**Host cells are more easily penetrated, cell death is delayed for 22 hr or more (6), and the vegetative portion of the fungus becomes well established. In a very susceptible variety, the fungus spreads rapidly over the infected potato surface.

Cytological studies of fungal penetration and cell death (7,8,9) have employed the epidermal cells of young leaves or cut surfaces of mature leaf petioles. In the present study, internal tissue from resistant and susceptible tubers was inoculated with the fungal spore suspension in order to study preinfection stages (spore germination, germ tube, and appressorium formation) and infection stages (intercellular and intracellular hyphal growth, haustorium formation, cell death) of fungal growth.

**See discussion with reviewers, p. 402.

Experimental

A sporangia suspension was obtained from fungal mats of P. infestans, race 4, grown on lima bean (10) or rye agar (11). Sterile water, 5 ml, was pipetted into the culture and sporangia were detached from hyphal tips by gently rubbing the fungal mat with a glass rod. If zoospores were desired, the suspension was placed in the cold (10 C) overnight, then brought to room temperature. Under these conditions, zoospores are ejected into the liquid medium. All experiments were conducted with race 4, and resistance and susceptibility relate to the reaction of the cultivar to race 4.

Clean, mature tubers of varieties Kennebec (resistant) and Houma (susceptible) were surface sterilized with 70% ethanol. Tubers were peeled and sliced into 3-5 mm slices. Each slice, in a sterile petri plate, was inoculated with 0.5 ml of an homogeneous suspension of spores and sporangia. Incubation was at 20 C in the dark. Infection was evidenced in the Kennebec by appearance of scattered necrotic areas on the surface of the slice, and in the Houma by obvious proliferation of the vegetative portion of the fungus on the slice. Control slices were slightly browned but clear of infection. Samples of the infected slices were cut out with a #2 cork borer, rinsed with water, and suitably trimmed (approximately 4x4x2 mm) with a razor blade. The samples were then fixed overnight in fresh 2% glutaraldehyde, 0.15 M phosphate buffered at pH 6. Fixed specimens were rinsed thoroughly, serially exchanged into absolute ethanol, and then into amyl acetate, and dried in a Denton* Critical Point Drying Apparatus from liquid carbon dioxide. Specimens were mounted on stubs with silver paint.

In order to obtain samples showing early stages of fungal development, a procedure was developed using light microscopy to monitor the potato tissue during incubation. Sections 200 μ thick and about 2 cm square were cut from varieties Houma (susceptible) and Kennebec (resistant). The sections were floated in sterile water to rinse out most of the starch granules, which interfere with microscopic examination of the potato cell wall. The subsequent sample handling and incubation were carried out under sterile conditions insofar as possible, to

* The mention of commercial items is for your convenience and does not constitute endorsement by the U. S. Department of Agriculture.

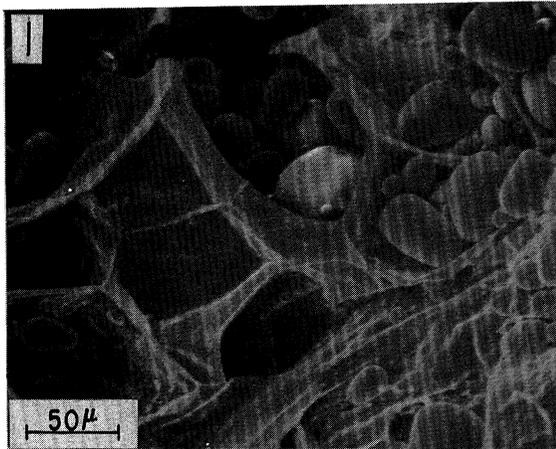


Figure 1. Surface of slice of Kennebec potato, critical point dried. Starch granules are visible in many cells.

minimize the growth of bacterial contaminants during incubation. Sterile water was pipetted onto microscope slides covering the surfaces to a depth of 1 or 2 mm. The potato sections were transferred to the slide on the tip of a microspatula. Each slide held one section each of Kennebec and Houma. Excess water was removed and the slide was positioned in a petri plate on a filter paper mat. One drop of sporangia suspension was placed on each section and a coverslip was applied, primarily to immobilize the tissue during incubation. The filter paper mat was saturated with water and the covered petri plate was incubated at 20 C.

The slides were examined periodically by light microscopy for evidence of fungal growth and selected sections were prepared for the scanning electron microscope. The coverslips were removed and the slides were cut into two pieces, each containing a potato section. Each piece was frozen in Freon 22 and transferred to a freeze drying apparatus. When dry, the sections maintained enough rigidity to enable easy trimming. Portions of each section were mounted directly on a stub and secured with silver paint at points on the periphery.

All mounted samples were made conductive with approximately 15 nm of gold-palladium alloy (60/40) on the rotary tilting stage of a Denton High Vacuum Evaporator, and were observed in a JSM-50A scanning electron microscope at 8 - 10 KV, with an objective aperture of 200 μ .

Results and Discussion

The 200 μ potato sections yielded much better micrographs than the larger 4x4x2 mm pieces. Because of the thin sections, secondary emission was uniform with little charge accumulation. Prior removal of starch granules improved image quality. The granules are not

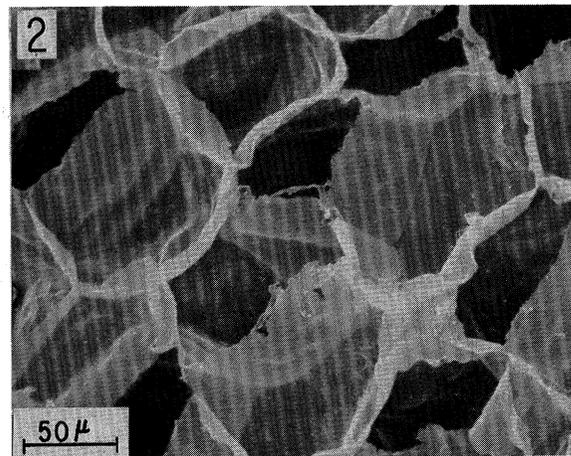


Figure 2. Surface of thin (200 μ) section of Kennebec potato, freeze dried. Starch granules have been washed out.

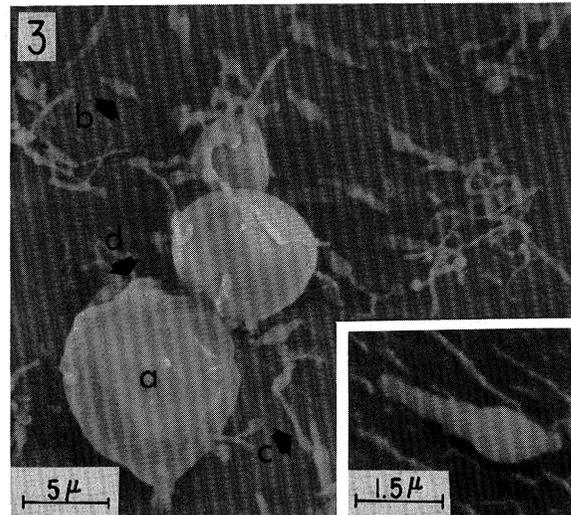


Figure 3. Sporangium (a) and zoospores [reniform (b) and tadpole-shaped (c and inset)]. Zoospores are expelled from the sporangium through the apical pore (d).

securely attached to the substrate and charge easily, often being dislodged from the sample by the electron beam. Figure 1 shows a normal surface of a slice of Kennebec potato which was fixed and critical point dried. Starch granules are visible in many cells. Figure 2 shows an area of a 200 μ Kennebec section which was freeze dried. The cell walls are well exposed for good observation.

On a Kennebec potato incubated for 24 hr (Figure 3), a sporangium (a), typically pear-shaped, is surrounded by many zoospores in various stages of early development. Most are reniform (b) while some appear tadpole-shaped

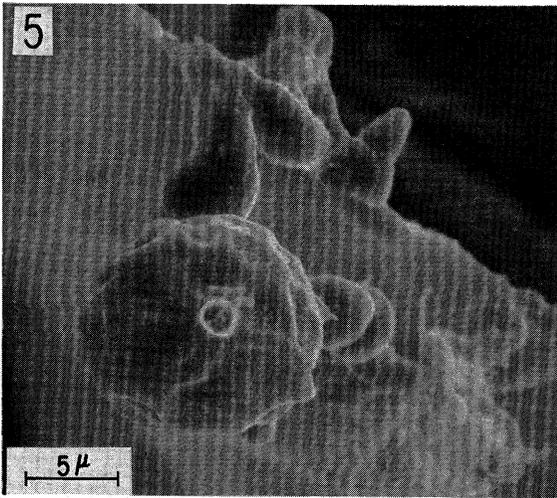
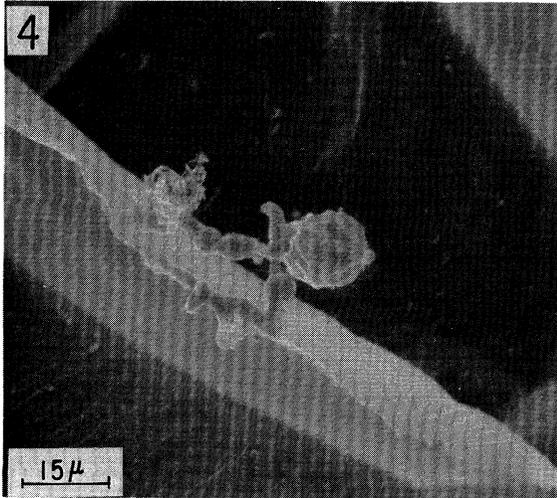


Figure 4. Germinating sporangium on vertical cell wall.

Figure 5. Germinating sporangium, showing surface structure. Stalk end of sporangium points toward viewer.

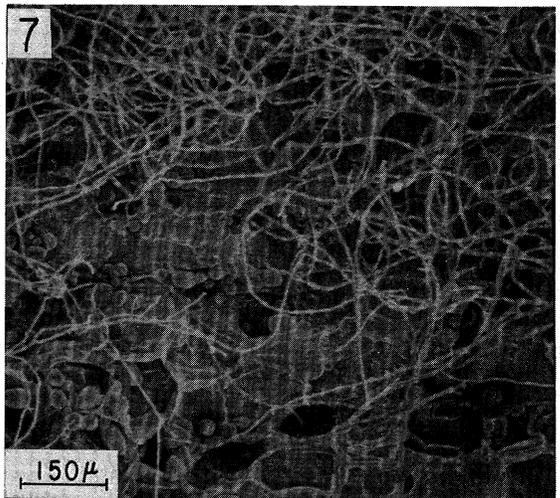
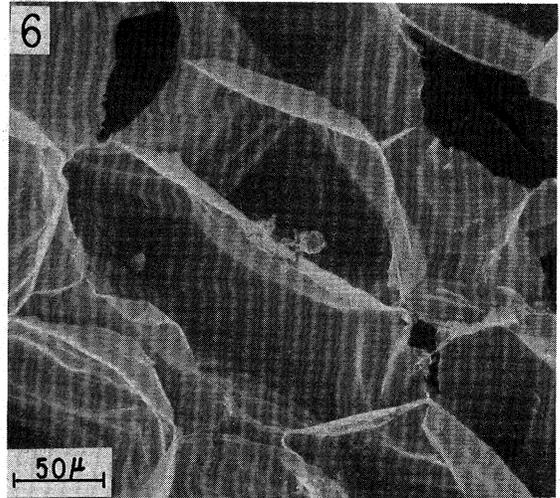


Figure 6. Potato cells with germinating sporangium.

Figure 7. Mycelium covering most of surface of infected Houma slice.

with head and cylindrical tail (c and inset). Zoospores develop within the sporangium and are expelled under favorable conditions through the apical pore (d). Two starch granules lie near the sporangium.

A sporangium may, instead of producing zoospores, germinate directly as seen in Figures 4 and 5. Rudimentary hyphae have developed from the apical end; the hyphae attach to a vertical potato cell wall on a Kennebec section. The germinating sporangium exhibits much more surface structure than would be inferred from observations by light microscopy. The difference may be accounted for by the transparency and edge imaging in a light microscope wet mount, and the greatly increased depth of field of SEM. A filamentous mass appears at the point

of attachment of one hypha. No similar structures were found elsewhere on the potato cell walls, and the overall surface of this sample was clear of cell debris (Figure 6). The mass may represent an accumulation of protoplasmic strands at the penetration site which was observed by Kitazawa *et al.* in light microscopy studies (9). Kitazawa found that masses always developed at infection sites in incompatible reactions, but only occasionally in compatible reactions.

The rate of growth of *Phytophthora infestans* on the thin sections varied greatly depending on the culture used for inoculation. In one series using susceptible cultivar Houma, after three days of incubation, hyphae emanated from many sporangia and germ tubes were present on many swollen zoospores. In a different

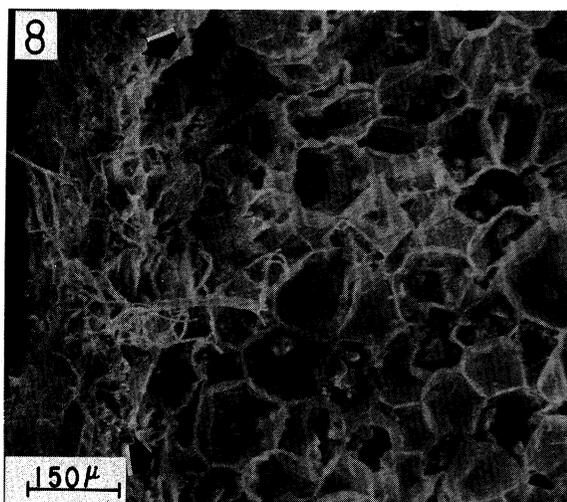


Figure 8. Cross section of infected Houma slice. Dense mycelium covers the inoculated surface, but does not extend beyond a 1 or 2 cell depth into the tissue (arrows).

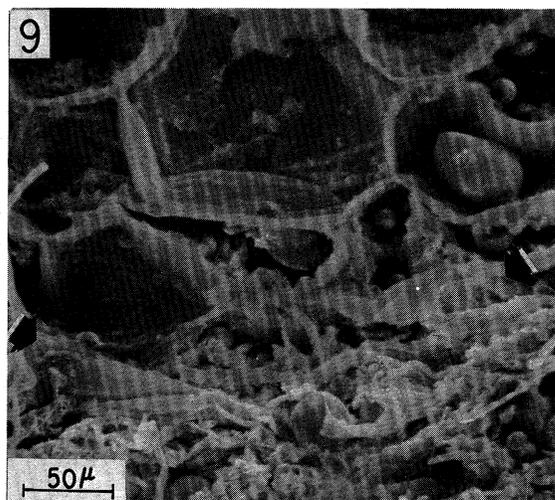


Figure 9. Outermost cells of infected Houma slice, showing massed hyphae near the surface and healthy cells beneath. Well-defined area delimits heavy hyphal growth (arrows).

inoculation series on Houma, fungal activity was much slower. Zoospores did not mature; sporangia germinated within a few hours of inoculation, but hyphal growth was nonexistent or slow, becoming evident only after a week or more of incubation.

Figure 7 shows a well-developed infection on the surface of a Houma slice. The hyphal growth is extensive and well branched. In the area shown, potato cells and starch granules are visible beneath the mycelium. In cross section (Figure 8) the infected surface (side of micrograph) is densely covered by mycelial mat. The outermost cells are seen to be packed with hyphae (Figure 9). However, an area of demarcation between infected cells and apparently healthy cells is readily identified (arrows, Figures 8 and 9). While the potato cells obviously provide nourishment for the fungus, the mycelial mat becomes thicker by growing outward from a surface, which maintains its structural integrity for a relatively long time, until rot overtakes the structure. There appears to be as little as a one cell barrier between active infection and intact cells. Infection of susceptible cultivars by *Phytophthora infestans* has been shown by cytological studies to proceed by intercellular hyphal growth. Haustoria or feeding hyphae, grow off the main hyphae into the cells without causing cell death. In a surface infected slice, intercellular hyphae eventually emerge from the other side, developing a hyphal mat which engulfs the entire slice.

Summary and Comment

Spore suspensions varied considerably in their abilities to germinate and infect. The

reasons for this were (a) natural variations in the fungus and (b) differences in the time allowed for the stock culture to grow out. The best cultures were from 1 to 2 weeks old. The light microscope was invaluable in determining the "potency" of the culture, *i.e.*, the rate of development of germ structures. This quality must be distinguished from virulence, or the ability to cause infection. Virulence is assessed by the response of the potato.

The success of *Phytophthora infestans* in parasitizing the susceptible potato is a consequence of its ability to infect without killing the cell immediately. Fig. 7 shows the colonization of a potato slice. A reservoir of intact cells remains to support the colony with nutrients by way of a network of intercellular hyphae. In hypersensitive resistance, cell penetration is followed rapidly by cell death; cell death is followed by fungal death. Well-defined causal relationships among these events have not yet been established.

The ability of the scanning electron microscope to provide high resolution imaging of fungal structures such as sporangia, zoospores, hyphae, as well as infection structures, such as the point of penetration into the cell and altered hyphal growth, makes it a valuable research tool in studying the infective process of *Phytophthora infestans*. Fungal structures can be located precisely with respect to the cell wall. The use of light microscopy in monitoring infection of the potato is a convenient way to generate samples containing maximum information for the scanning electron microscope.

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Acknowledgment

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DISCUSSION WITH REVIEWERS

Author's Late Addition: Additional reference - D.D. Clarke, "The Resistance of Potato Tissue to the Hyphal Growth of Fungal Pathogens," *Proc. R. Soc. Lond. B.* 181, 1972, 303-317.

Reviewer I: Are the zoospores really ejected?

Authors: We have seen them ejected under the light microscope. How they get out depends on the environmental conditions at the time. We have also seen the zoospore mass clinging to the sporangium at the pore.

Reviewer III: Were all tissue slices incubated in the dark?

Authors: Yes.

Reviewer II: Why was critical point drying used for larger samples and freeze drying for smaller ones? It is difficult to compare the two preparation procedures as there are differences in size, washing and dehydration procedures.

Authors: Freeze drying was selected for the 200 μ sections for ease of handling. The delicate wet section mounted on slides could be frozen and dried right on the slide. Preliminary experiments had indicated that both CPD and freeze drying were acceptable techniques for potato tissue.

Reviewer I: Early states of zoospore development would correspond to their development within the sporangium. I would suggest that Fig. 3 represents various stages of germination of cysts (encysted zoospores).

Authors: We believe that the structures in question are the motile zoospores. Structures believed to be cysts have occasionally been observed by us both by SEM and light microscopy, but more after the smaller zoospores are present in abundance. Those in Fig. 3 are larger than those which have just emerged from sporangia.

DISCUSSION WITH REVIEWERS (CONT'D)

Reviewer III: Is the apical pore (Fig. 3) really visible?

Authors: Yes. After the sporangium empties, the apical pore is a hole which is visible, as shown in Fig. A. There are two sporangia in the photo, one with the stalk end, one with pore end, toward viewer.

Reviewer I: How long after inoculation was material for Fig. 7 fixed?

Authors: Five days.

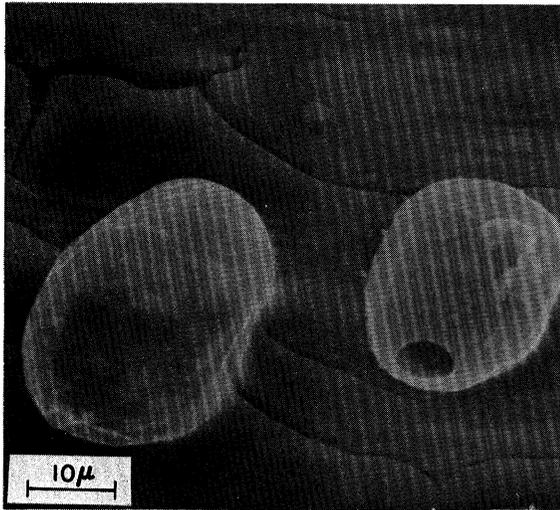


Fig. A - Sporangia. Stalk end of one (left), and apical pore end of the other (right), are visible.

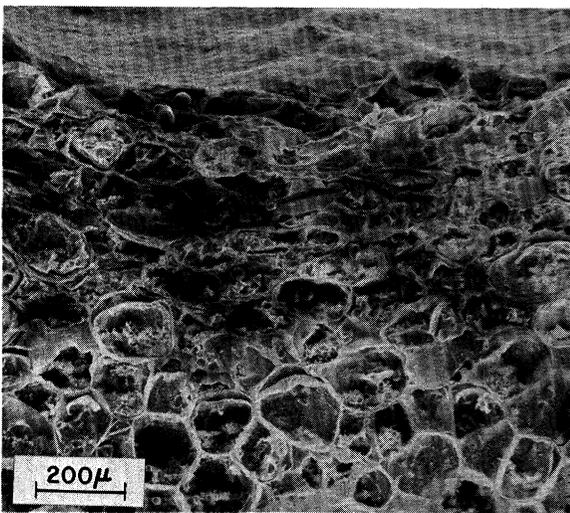


Fig. B - Cross section of necrotic area of Kennebec potato slice. Dead cells are collapsed, appearing flattened in micrograph.

Reviewer I: Does this study with the SEM provide any additional information to those cytological studies involving light or transmission electron microscopy?

Authors: Information obtained in this study complements light and transmission electron microscopy. Unique information has been obtained concerning a) appearance of hyphal surface, b) growth characteristics of hyphal tip, c) appearance of area of penetration, d) appearance of physical attachment to cell wall, and e) growth patterns of hyphae with respect to cell wall.

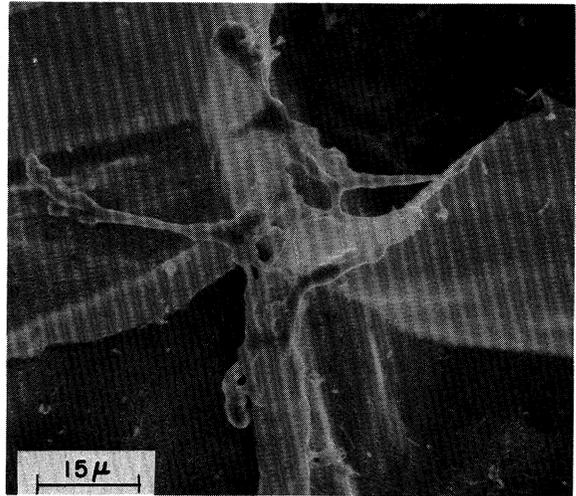


Fig. C - Hyphal mass affixed to potato cells at junction of four cells.

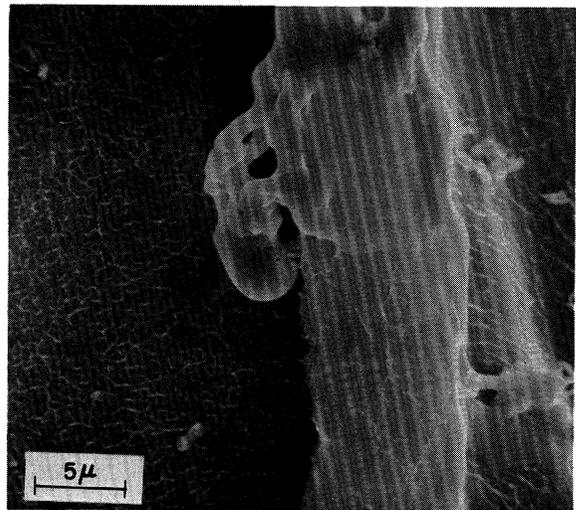


Fig. D - Enlarged area of Fig. C showing substance which appears mucilaginous covering hypha and area of cell wall.

DISCUSSION WITH REVIEWERS (CONT'D)

Reviewer I: Is it possible to recognize haustoria within the potato cells?

Authors: Haustoria should be visible by SEM. We have not as yet seen this structure.

Reviewer I: By using SEM, can various stages in the hypersensitive reaction of host cells be determined?

Authors: Stages of cell death which involve structural changes are visible after cell death, the cell walls eventually fail, giving the cells of the necrotic area a flattened appearance when viewed in cross section (Fig. B). Other changes, such as lesions of the cell wall around a penetrating hypha, suggest themselves, but the findings are preliminary at this time.

Reviewer I: With many other fungi, the penetrating hyphae appear to secrete a mucilaginous substance which holds them to the host surface. Is this also the case with P. infestans?

Authors: We have observed mucilaginous material associated with some areas of hyphal growth, for example, the hyphal mass at the corner of 4 cells shown in Fig. C. In the enlargement (Fig. D), the hyphae and nearby cell wall are covered by a smooth layer which appears mucilaginous.

Reviewer III: In your experimental section, you speak of evidence of infection on both Kennebec and Houma. In the Kennebec, was it infection that was evident, or resistance? In Houma, did the fungus establish itself or was it there without infection?

Authors: Resistance in various cultures is a matter of degree, ranging, theoretically, from none to total. In total resistance, there would, theoretically, be no infection. In hypersensitive resistance, cell penetration (infection) by the fungus elicits a resistant response. In that sense, resistance in Kennebec is evidence of (limited) infection. In the case of Houma, the rapid growth of fungus on the surface implies a supply of nutrients from the potato, surely this is infection.

Reviewer IV: Have you tried such controls as killing Kennebec cells or washing them for long periods of time to eliminate, possibly, the substrates involved in the resistance phenomenon? They might become susceptible.

Authors: Biochemical studies are underway to examine the molecular phenomena involved in the resistant and susceptible interactions.