

## Perforation and Destruction of Pigmented Hyphae of *Gaeumannomyces graminis* by Vampyrellid Amoebae from Pacific Northwest Wheat Field Soils

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### ABSTRACT

HOMMA, Y., J. W. SITTON, R. J. COOK, and K. M. OLD. 1979. Perforation and destruction of pigmented hyphae of *Gaeumannomyces graminis* by vampyrellid amoebae from Pacific Northwest wheat field soils. *Phytopathology* 69:1118-1122.

Vampyrellid amoebae were isolated from two Pacific Northwest wheat field soils by burying pigmented hyphae of *Gaeumannomyces graminis* var. *tritici* as bait for 4-8 wk. The amoebae caused holes (average diameter 1.7  $\mu$ m) and numerous annular depressions (incomplete holes) in the hyphae. The annular depressions, holes, and the amoebae that caused them

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all matched those previously described from Scotland and Canada. The amoebae caused perforations of conidia of *Cochliobolus sativus* in water cultures. The time from initial contact of a hyphal cell by the pseudopodium of an amoeba until the cell was empty of contents ranged from 40 to 90 min.

Old (5), Old and Darbyshire (6), and Anderson and Patrick (1) working in Scotland and Canada, respectively, recently demonstrated that perforations in the pigmented walls of spores of various fungi buried in soil are caused by vampyrellid amoebae. The amoebae from soils in Scotland were identified as *Arachnula impatiens* Cienk. (6), and those from soils in Canada as possibly *Biomyxa vagans* Leidy (1). Both genera are members of the family, Vampyrellidae Doflein (4).

*Gaeumannomyces graminis* (Sacc.) Von Arx and Olivier var. *tritici* Walker, cause of take-all of wheat (*Triticum aestivum* L.) uses pigmented hyphae (runner hyphae) to attack wheat roots. These pigmented hyphae spread ectotrophically along the root surface and from root to root, and play an important role in the persistence of the fungus in the rhizoplane of wheat. The fungus also survives saprophytically in host residue as pigmented hyphae. Soil organisms that interfere with the growth or health of pigmented hyphae of *G. graminis* var. *tritici* should have potential for biological control of take-all.

Although much is known about the effects of cultural practices on take-all, little is known of the specific kinds of microbiological interactions between *G. graminis* var. *tritici* and associated microbiota in soil, on wheat roots, or in host residue. The discovery that amoebae perforate pigmented spores and the fact that *G. graminis* var. *tritici* forms pigmented hyphae during both parasitism and saprophytism prompted us to question whether amoebae could perforate and kill pigmented hyphae of the take-all fungus. Rovira and Campbell (8) observed holes in the hyphae of *G. graminis* var. *tritici* on roots of wheat and suggested that they were similar to those reported for pigmented spores. However, their observations preceded the discovery that amoebae cause holes in pigmented spores. Of equal relevance is whether the amoebae able to perforate fungal cells are unique to soils in Scotland (5,6) and Canada (1) or if they are widespread in nature. This study was undertaken to determine whether vampyrellid amoebae capable of

perforating pigmented cells of fungi occur in wheat-field soils in the Pacific Northwest, and whether they can cause perforations and death of the pigmented hyphae of the wheat take-all fungus, *G. graminis* var. *tritici*.

### MATERIALS AND METHODS

**Soils.** Soils were collected from two wheat fields, one near Moses Lake, Washington, and the other near Quincy, Washington. Both fields are in the Columbia Basin and had been planted to wheat for about 20 consecutive years at the time soil was collected. Soil from the field near Moses Lake was a clay loam and that from near Quincy was a silt loam. Each soil was dried slowly, blended, passed through a 4.0-mm (5-mesh) sieve, and then stored in galvanized cans in an unheated shed. The Quincy field had been the source of soil for earlier investigations of the factor(s) responsible for take-all decline in the Pacific Northwest (3). Soil was collected in September 1977 for studies from October 1977 through March 1978. Fresh soil was collected in April 1978, for studies through September 1978.

**Production of pigmented hyphae of *G. graminis* for addition to soil.** Pigmented hyphae of *G. graminis* were used as bait to isolate amoebae from soil. Disks of cellophane were cut to fit inside a 9-cm-diameter petri dish. The disks were cleaned for 10-15 min in boiling water, autoclaved, and then placed on the surface of 1/5th-strength potato dextrose agar in petri dishes. An agar plug (5 mm in diameter) from the advancing margin of a culture of *G. graminis* was placed on the cellophane in the center of each dish. An appressed colony of the fungus grew superficially over the cellophane and the hyphae developed the typical dark pigment after 3-4 wk at 24 C. At that time, the cellophane was sectioned into squares 0.5  $\times$  0.5 cm with a sharp, sterile scalpel and each square was transferred with a forceps to sterile distilled water in which the mycelium floated free from the cellophane. The mycelium was transferred through several changes of distilled water to remove residual nutrients from the medium and each mat was placed on a 13-mm-diameter Millipore filter (pore size, 1.2  $\mu$ m). The Millipore filter was sandwiched between two 25-mm-diameter Nuclepore

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filters (Nuclepore Corporation, Pleasanton, CA 94566) with openings 1  $\mu\text{m}$  in diameter (5). The Nuclepore filters were sealed together at the margins with high-vacuum grease and these "sandwiches" were buried in moist ( $\sim 100$  mb) soil in petri dishes with lids perforated for aeration. After incubation for 4–8 wk at 25 C, the hyphae were examined, then used for isolation of the amoebae.

**Tests for the presence of amoebae.** To culture the amoebae, a portion or all of a Millipore filter with adhering hyphae and associated microorganisms from soil was placed in a shallow layer (1–2 mm deep) of distilled water or 10% soil extract (1) in a 55-mm-diameter glass petri dish. Conidia of *Cochliobolus sativus* Ito and Kuribayashi (Drechsler ex Datur) were added to the water as fresh food for the amoebae. Subcultures were prepared after 1 wk of incubation in the dark at 25 C by transferring a 1- to 2-ml suspension of the water culture to fresh distilled water or 10% soil extract with fresh spores of *C. sativus*.

Other Millipore filters or the remaining portions of those used to start cultures were prepared for examination either by bright-field, phase-contrast, or scanning electron microscopy (SEM). Specimen preparation for light microscopy was as described by Old and Patrick (7). For preparation of specimens for SEM, the hyphae were fixed for 1 hr in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), dehydrated in a graded ethanol series, and passed through a series of ethanol and Freon 113 to pure Freon 113. They were then critical-point dried in a Bomar critical-point drying apparatus (2). Dried specimens were mounted on stubs, coated with a 15-nm layer of gold, and examined in an ETEC Autoscan scanning electron microscope.

For estimations of populations of amoebae, the individual soils were suspended in sterile water, diluted  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$ , and then 1 ml of each dilution was added to 1 ml of soil extract solution in test tubes together with a standardized suspension of conidia of *C. sativus* (1). Five replicate dilution series were prepared for each soil. After 4 wk, a few drops of the suspension was transferred from each tube to a glass slide, the number of perforated conidia were counted by microscopic examination, and the population of amoebae then was estimated by most probable number (1).

**Direct observations of amoebae attacking hyphae of *G. graminis*.** Direct observations of amoebae attacking hyphae of *G. graminis* were made under phase-contrast microscopy at  $\times 600$ . Cellophane squares with superficial hyphae of *G. graminis* were placed in a 55-mm-diameter petri dish containing distilled water or 10% soil extract and seeded with trophozoites (individuals in the active amoeboid form) and cysts of amoebae from a culture started with hyphae from soil. After 3–4 days at 25 C, the cellophane pieces were placed on a glass slide. Wet filter papers were also placed on each slide in contact with each side of the cellophane and used as wicks to keep the cellophane moist. The filter papers were watered with distilled water or 10% soil extract frequently during observation. Photographs were taken every 30 min.

## RESULTS

**Observations of hyphae of *G. graminis* recovered from soil.** Annular depressions and perforations were observed on all pigmented hyphae recovered from either soil (Fig. 1A and B). The annular depressions and perforations were observed as soon as 4 wk after burial, but maximal perforation was not evident until 6–8 wk after burial. The annular depressions began as slight etchings in the hyphal wall, and became progressively deeper until the hyphal wall was perforated and the resultant disk of wall material was gone. The average diameter of the perforations was 1.7  $\mu\text{m}$  (1.2–2.2  $\mu\text{m}$ ), with one to three holes per hyphal cell (Fig. 1A). Holes less than 0.1  $\mu\text{m}$  in diameter also were observed in the walls. Bacteria, fine hyphae (probably actinomycetes), and cysts of various sizes were observed under the SEM in association with the hyphae of *G. graminis*. Presumably, these organisms or their parents passed through the 1- $\mu\text{m}$ -diameter pores of the Nuclepore filters.

**Isolation of amoebae from soils.** Trophozoites with numerous

extended filopodia and similar to those described by Old (5) and by Anderson and Patrick (1) were observed within 3–4 days after mycelium of *G. graminis* from either of the two soils was placed in water containing spores of *C. sativus*. Amoebae similar to *Leptomyxa reticulata* Goodey, and one or two other unidentified amoeboid species also were observed. Nearly pure culture of vampyrellid amoebae were obtained after several subcultures from the original culture. This was possible because the vampyrellid amoebae were much more common than the other amoebae. However, as reported by others (6), we were unable to obtain cultures free of bacteria.

At soil dilutions of  $10^{-1}$ , and with conidia of *C. sativus* added as the bait organism, the percentage of perforated conidia after 4 wk was 5.3 and 57 for the soils from Moses Lake and Quincy, respectively. At  $10^{-2}$ , perforated conidia were detected in only one of the five samples of soil from the field near Moses Lake and two of the samples of soil from the field near Quincy. Based on the counts and the most probable number method, we estimated the populations of amoebae responsible for perforations of spores at about 20 and 70 per gram in the soils from Moses Lake and Quincy, respectively.

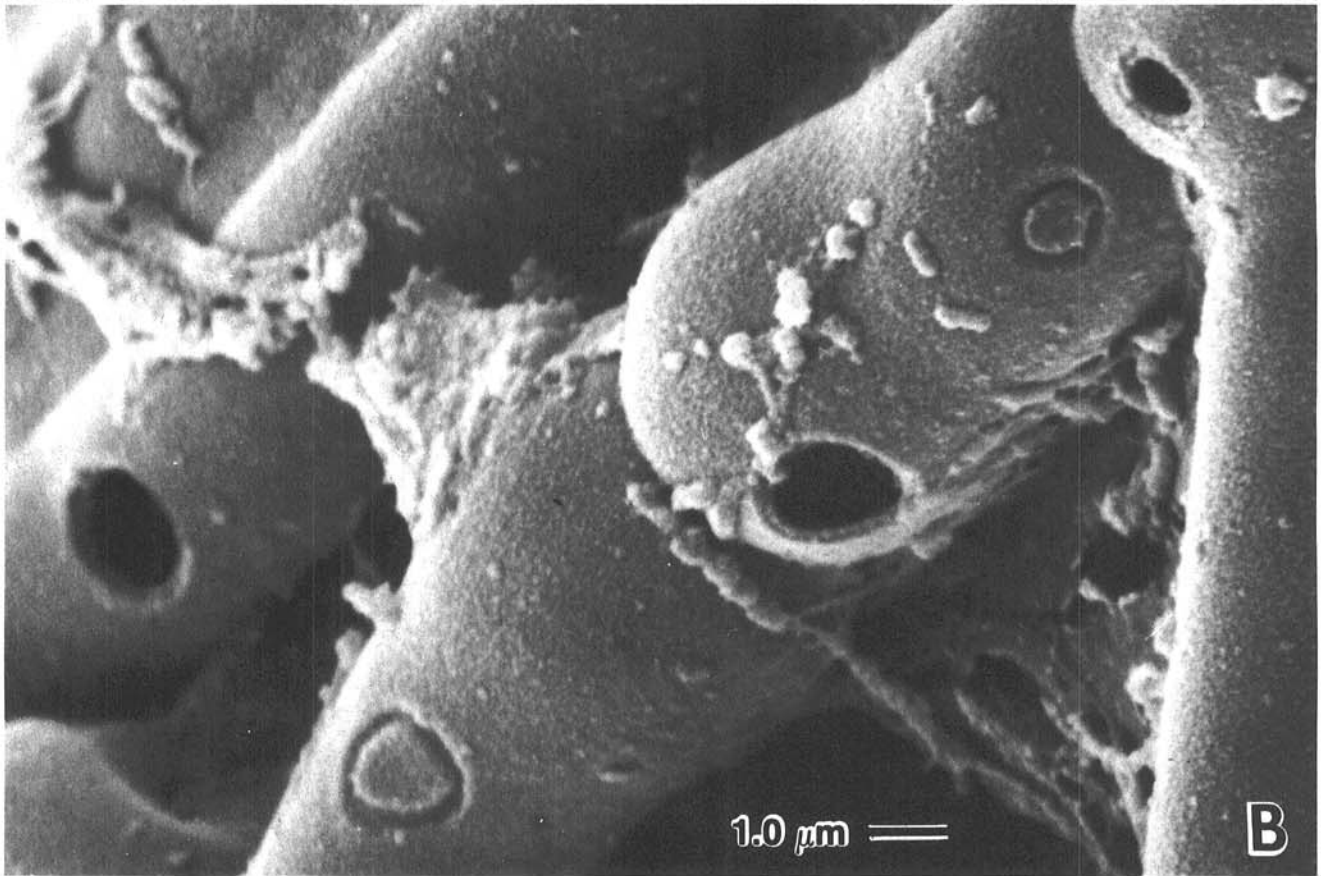
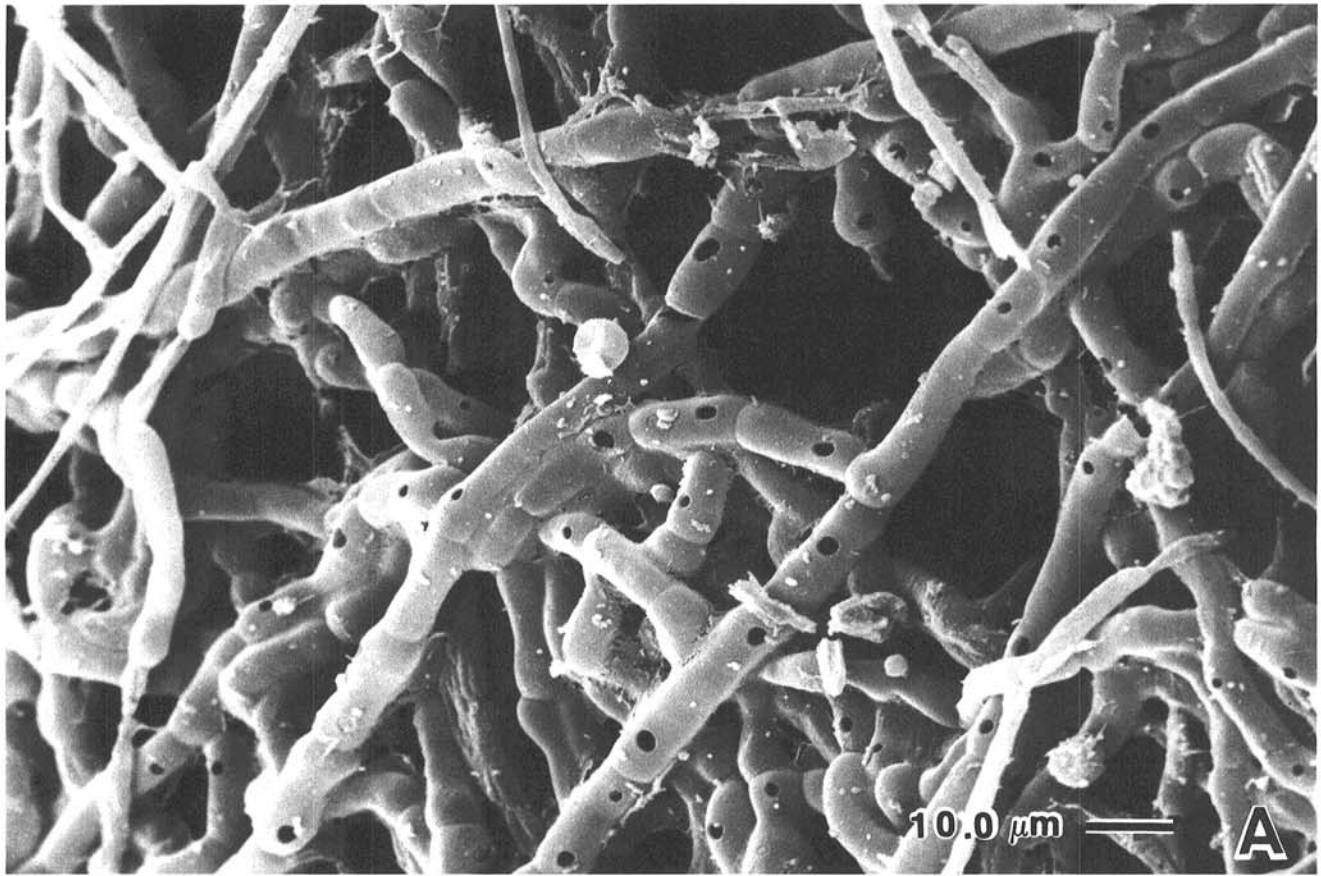
**Direct observation of the perforation process.** Only the vampyrellid amoebae with the fine filopodia perforated pigmented hyphae in the water culture. Moreover, these amoebae only produced the larger holes (1.2–2.2  $\mu\text{m}$ ) and not the holes smaller than 0.1  $\mu\text{m}$ . In some cases, an amoeba covered the hypha completely. Cytoplasmic movement was observed within hyphal cells within 10–20 min after contact between an amoeba and a hyphal cell. Most contents of the hypha usually disappeared within 30 min after amoeba-hypha contact, leaving only granular material within the hyphal cell. This granular material then moved in the direction of the amoeba protoplasm, and after 40–90 min the hyphal cell was completely empty. As the amoeba moved away from the empty cell, the fine filopodia often could be observed within the empty hypha. After the amoeba had left the hypha, generally one to three holes could be observed in the wall of each empty cell. *Leptomyxa reticulata* also was observed in association with hyphae in water culture, but did not cause holes.

## DISCUSSION

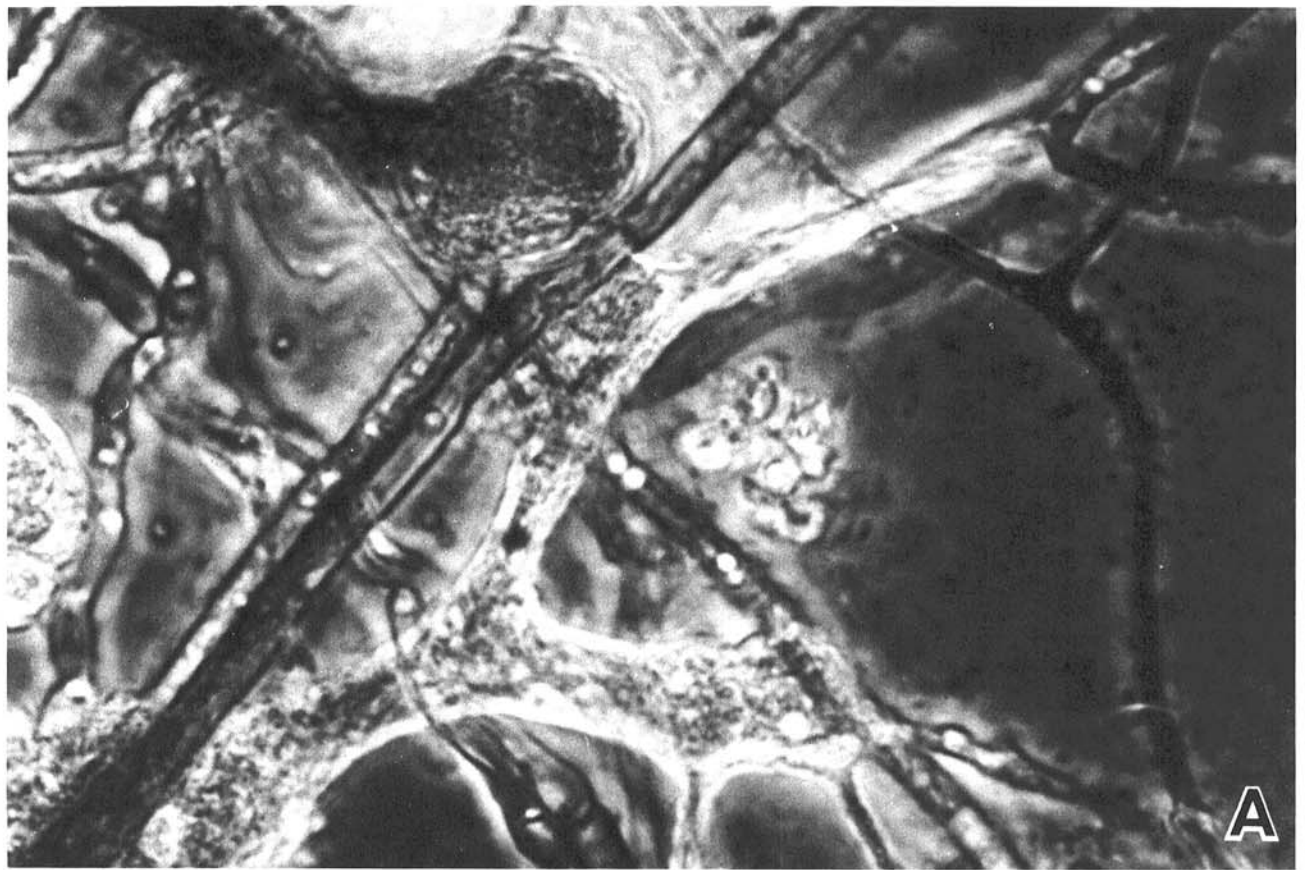
Vampyrellid amoebae capable of causing annular depressions and perforations of pigmented hyphae of *G. graminis* var. *tritici* were recovered from both of the Pacific Northwest wheat field soils that were tested. Perforations in hyphae were evident as early as 4 wk after burial, and by 6–8 wk virtually every hypha was perforated at least once and some were perforated many times. Examination of perforated cells by phase contrast microscopy and direct observation of the perforation process revealed that all cell contents were removed and that the cell wall was left as an empty cylinder.

The amoeba that perforated the pigmented hyphae of *G. graminis* var. *tritici* also caused holes, identical to those described by Old (5), in spores of *C. sativus*. The time from initial contact of an amoeba and a hyphal cell until the cell was empty was about 40–90 min; Old (5) observed that about 4.5 hr was required for perforation of conidia of *C. sativus* and Anderson and Patrick (1) observed that 15–60 min were required for perforation of chlamydospores of *Thielaviopsis basicola*. Differences of this magnitude should be expected with different cultural conditions and fungus species. Thus, it appears that the amoebae that perforate pigmented hyphae of *G. graminis* var. *tritici* are the same or very similar to the vampyrellid amoebae reported in Scotland (5) and in Canada (6). That, and the recovery of these amoebae from both soils that were tested and the demonstration that they cause perforation of hyphae of the take-all fungus suggest that these animals are ubiquitous in nature and relatively nonselective in their feeding. These conclusions support the suggestion of Old and Darbyshire (6).

The cause of the perforations smaller than 0.1  $\mu\text{m}$  is still unknown; Old (5) and Old and Darbyshire (6) observed similar tiny holes in the walls of pigmented spores recovered from soil. It is



**Fig. 1.** Photomicrographs of annular depressions and perforations of hyphae of *Gaemannomyces graminis* var. *tritici* caused by vampyrellid amoebae following burial for 8 wk in soil. **A**, Several hyphae showing one to three perforations per cell. **B**, Larger magnification of perforation and annular depressions.



**Fig 2.** Phase-contrast view of a trophozoite of a vampyrellid amoeba responsible for perforation and emptying of the cell of a hypha of *Gaemannomyces graminis* var. *tritici*. **A**, View 30 min and **B**, 60 min after the trophozoite made contact with the hyphae. Note the digestive cysts of other specimens of vampyrellid amoebae.

unlikely that the vampyrellid amoebae are responsible for the small holes, but the role of other amoebae and of bacteria cannot be ruled out.

The taxonomy of the amoeba or amoebae responsible for the large perforations is uncertain at present. *Arachnula impatiens* and *Biomyxa vagans* are closely related (4) and may be the same species; both produce fine filopodia and both have long been recognized to feed on algae and diatoms. We have assigned the amoeba responsible for perforations of the hyphae to *A. impatiens* since the specimens are indistinguishable from those described by Old and Darbyshire (6).

#### LITERATURE CITED

1. ANDERSON, T. R., and Z. A. PATRICK. 1978. Mycophagous amoeboid organisms from soil that perforate spores of *Thielaviopsis basicola* and *Cochliobolus sativus*. *Phytopathology* 68:1618-1626.
2. COHEN, A. C. 1974. Critical point drying. Pages 44-112 in Hyatt, ed. *Principles and Techniques of Scanning Electron Microscopy*, Vol. I. Van Nostrand, New York. 361 pp.
3. COOK, R. J., and A. D. ROVIRA. 1976. The role of bacteria in the biological control of *Gaeumannomyces graminis* by suppressive soils. *Soil Biol. Biochem.* 8:269-273.
4. KUDO, R. R. 1966. *Protozoology*, 5th ed. Chas. C. Thomas, Fort Lauderdale, Florida. 1,174 pp.
5. OLD, K. M. 1977. Giant soil amoebae cause perforation of conidia of *Cochliobolus sativus*. *Trans. Br. Mycol. Soc.* 68:227-281.
6. OLD, K. M. and J. F. DARBYSHIRE. 1978. Soil fungi as food for giant amoebae. *Soil Biol. Biochem.* 10:93-100.
7. OLD, K. M. and Z. A. PATRICK. 1976. Perforation and lysis of spores of *Cochliobolus sativus* and *Thielaviopsis basicola* in natural soils. *Can. J. Bot.* 54:2798-2809.
8. ROVIRA, A. D. , and R. CAMPBELL. 1975. A scanning electron microscope study of the interactions between microorganisms and *Gaeumannomyces graminis* (syn. *Ophiobolus graminis*) on the roots of wheat. *Microbiol. Ecol.* 2:177-185.