

# Pathogenesis of *Plasmopara lactucae-radialis*, a Systemic Root Pathogen of Cultivated Lettuce

M. E. STANGHELLINI, J. E. ADASKAVEG, and S. L. RASMUSSEN, Department of Plant Pathology, University of Arizona, Tucson 85721

## ABSTRACT

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*Plasmopara lactucae-radialis*, a recently described causal agent of downy mildew, is reported for the first time as a root pathogen of cultivated lettuce. This unique fungus, which only colonizes roots, caused yield reductions of about 50% in greenhouse pathogenicity tests. Direct penetration of roots by zoospore cysts, systemic invasion of roots by intercellular hyphae and intracellular haustoria, and sporulation on infected roots occurred at root temperatures between 18 and 26 C. No infection occurred at root temperatures of 12, 14, and 30 C. Lettuce cultivars *Ostinata* and *Salina* are susceptible and *Sitionia* is resistant.

Additional keywords: disease development, histology, ultrastructure, zoospores

The destructive nature of downy mildew of lettuce (*Lactuca sativa* L.), a foliar disease caused by *Bremia lactucae* Regel, is well documented (6). In 1987, a new causal agent of downy mildew, *Plasmopara lactucae-radialis* Stang. & Gilbn., was discovered on lettuce cultivars *Ostinata* and *Salina* in a commercial hydroponic greenhouse in Rapidan, Virginia, that employed the nutrient film technique of cultivation (Fig. 1A,B,C) (7). This fungus, in contrast to *B. lactucae*, was apparently restricted to the root system, which showed varying degrees of necrosis. Sporangia of the obligate parasite were microscopically observed only on roots of lettuce plants (Fig. 1D,E), which were of all ages and had been randomly collected throughout the 2.4-ha facility. Other organisms also were associated with the necrotic roots. *Pythium dissotocum* Drechs., a known subclinical root pathogen of hydroponically grown lettuce (8), was consistently isolated from lettuce roots, and microscopic examination of roots also revealed the presence of *Olpidium brassicae* (Woronin) Dang.

The occurrence of *P. lactucae-radialis* on the roots of lettuce is only the second confirmed report of a downy mildew fungus on roots. Another species of *Plasmopara*, *P. helianthi* Novot. f. *helianthi* Novot., is also known to sporulate on roots but primarily infects the foliar portions of its host, annual sunflower (2,3,6). The objectives of this investigation were to: 1) determine if root necrosis and yield reductions occurred as a consequence of root infection by *P. lactucae-radialis*, 2) determine the influence of temperature on disease development, 3) provide information on penetration, colonization, and sporulation of *P. lactucae-radialis* on lettuce roots, and 4) perform cross-inoculation studies between *P. lactucae-radialis* and *P. h. f. helianthi* on lettuce and sunflower.

## MATERIALS AND METHODS

Pure cultures of *P. lactucae-radialis*, an obligate parasite, were initially obtained from, and subsequently maintained on, lettuce plants. Unless otherwise specified, all studies were conducted on the cultivar *Ostinata*. Roots from naturally infected *Ostinata* plants, obtained from the commercial greenhouse in Virginia, were rinsed in running tap water for 3 min, blotted dry, and plated on the surface of water agar. Sporangiohores bearing sporangia were produced aerially on the surface of infected roots within 24 hr of incubation at 25 C. Sporangia were physically removed with a glass needle

and placed in small vials containing 1 ml of sterile distilled water. A 2-day-old lettuce seedling, germinated on moist filter paper, was then placed in each infested vial. After a 4-hr incubation period, during which zoospore release from sporangia was observed microscopically, seedlings were removed and placed in growth pouches (Northrup King, Minneapolis, MN). Growth pouches containing inoculated seedlings were then incubated (12-hr light cycles, 5,200 lx) in a growth chamber at 25 C. Roots of seedlings in growth pouches were examined daily. Infected seedlings, ascertained by the observation of sporulation on roots, were removed and transplanted into a hydroponic system in the growth chamber. Healthy lettuce seedlings were rotated into the system every 3-4 wk in order to maintain stock cultures of the fungus.

**Effect of temperature on infection.** Pathogenicity tests were conducted in a greenhouse under hydroponic conditions as previously described (8). Four 2-wk-old lettuce seedlings, started in a nursery in Oasis horticultures (Smithers Oasis, Kent, OH), were transferred into holes cut into Styrofoam flotation boards. Boards were then placed in plastic tubs containing a continuously aerated nutrient solution. Tubs were located in a temperature-controlled box, and the nutrient solution was equilibrated to 12, 14, 18, 26, and 30 C before the seedlings were transplanted (8). One week after transplanting, one lettuce plant infected with *P. lactucae-radialis* was added to each tub. Plants in noninfested tubs served as controls. After 4 wk of growth in the tubs, the shoots were weighed and the fresh weights were recorded for each treatment. All treatments were replicated four times (with four plants per replicate) and the experiment was repeated two times at each temperature. All temperatures, unless otherwise specified, refer to those of the hydroponic nutrient solution. Ambient temperatures in green-

house studies ranged daily from 23 to 34 C (mean, 28 C).

**Effect of temperature on sporangiophore formation and zoospore release.** Roots infected with *P. lactucae-radicis*, excised from 3-wk-old hydroponically grown plants, were washed in running tap water for 10 min, blotted dry, and placed on the surface of water agar contained in petri plates. Each plate contained 10 root segments, each 50 mm long. Plates were then incubated at 10, 15, 20, 25, and 30 C. The number of sporangiophores produced per 50 mm of root was recorded after 6, 24, 36, and 48 hr of incubation. Each treatment was replicated twice and repeated once.

Roots bearing sporangia from the above study at 25 C were removed from the agar surface and placed into preincubated petri dishes containing 10 ml of sterile distilled water. The plates were then immediately placed back into incubators at 10, 15, 20, 25, and 30 C. Zoospore release was monitored every 15 min over a 180-min period. At the end of each time interval, the number of zoospores produced was estimated as previously described (4). Each treatment was replicated twice and repeated once.

**Effect of plant age on susceptibility.** A plant infected with *P. lactucae-radicis* was transplanted into a tub containing four healthy plants 1–4 wk old. Portions

of the root system from each plant were excised periodically over a 2-wk period and: 1) microscopically examined for the presence of sporangiophores and sporangia, 2) stained with acid fuchsin and microscopically examined for hyphae and haustoria within root tissues, and 3) rinsed in running tap water, blotted dry, and plated on water agar. After 24 hr of incubation at 25 C, roots on agar were microscopically examined for the presence of aerial sporangiophores and sporangia. The experiment, conducted in the greenhouse at 26 C, was replicated twice and repeated twice. Any root colonization by *P. lactucae-radicis* was regarded as a susceptible reaction.

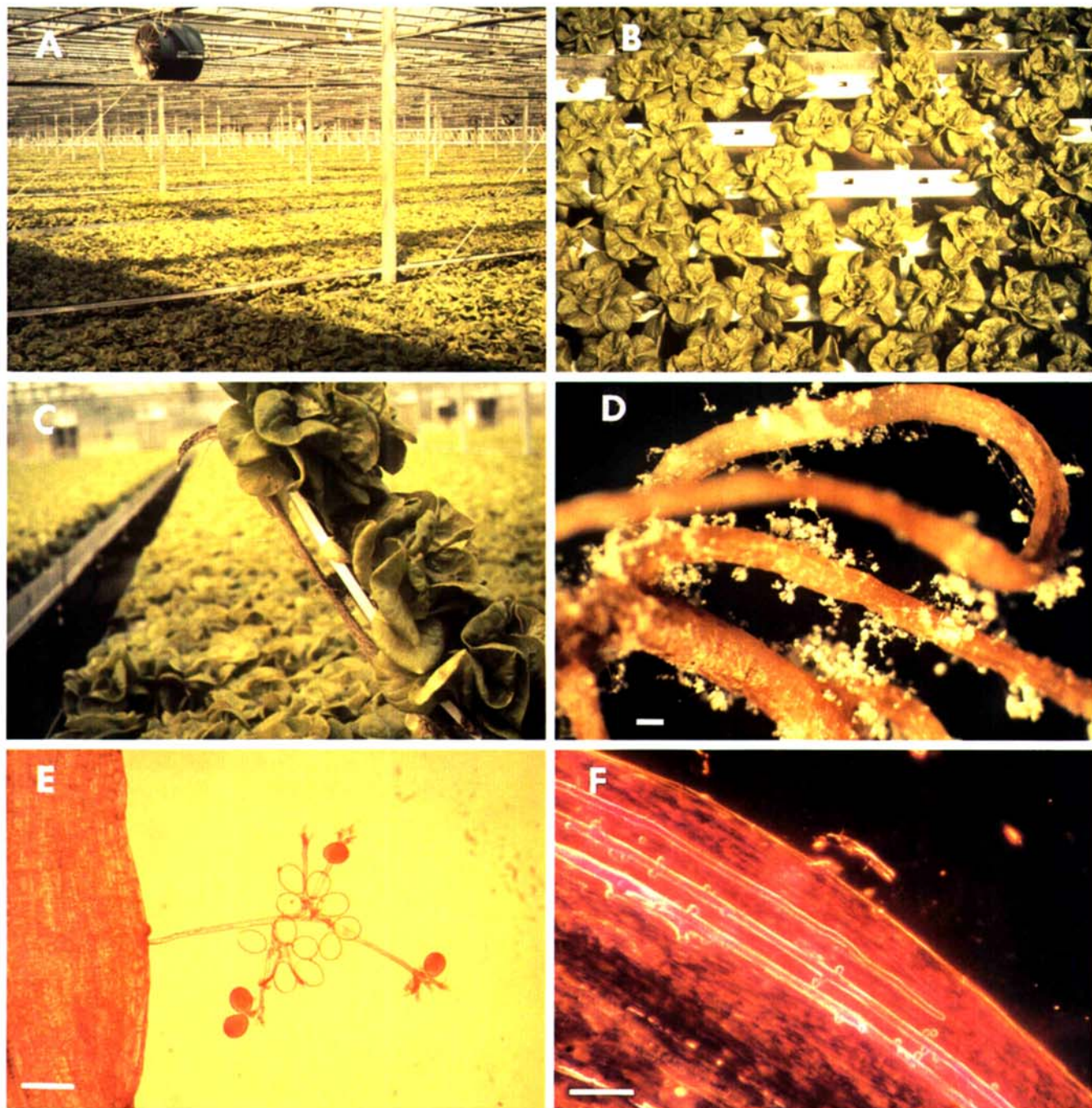
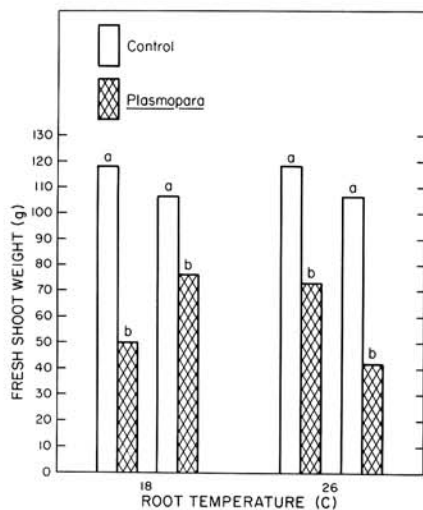


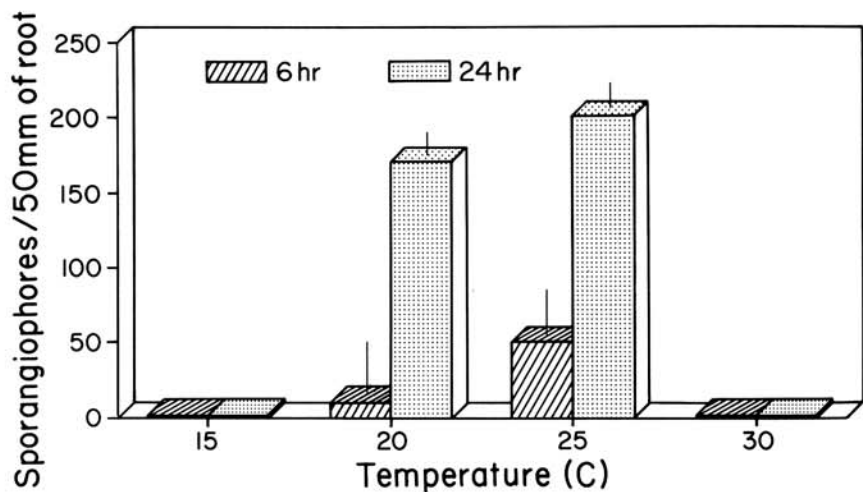
Fig. 1. (A,B,C) Hydroponically grown lettuce in a 2.4-ha greenhouse in Virginia employing the nutrient film technique of cultivation. (D,E) Sporulation of *Plasmopara lactucae-radicis* on infected lettuce roots. Scale bars = 1 mm on D and 100  $\mu$ m on E. (F) Intercellular hyphae and haustoria in the cortex of infected roots. Scale bar = 100  $\mu$ m.

**Scanning electron and light microscopy.** At time intervals of 15, 30, 60, and 120 min after inoculation with zoospores of *P. lactucae-radicis*, lettuce roots were prepared for microscopic examination. For scanning electron microscopy, excised root segments (5–10 mm long) were placed in an aqueous solution of 4% formaldehyde and 1% glutaraldehyde in a 0.01 M PO<sub>4</sub> buffer (pH = 7.2) for 24 hr, washed three times in distilled water for 15 min per wash, postfixed in an aqueous solution of 2% OsO<sub>4</sub> for 2 hr, and washed in distilled water as in the previous step. Samples were then placed in an alcohol dehydration series of 20, 50, 80, 95, 100, 100, and 100% ethanol for 15–30 min per treatment. Root samples for surface observations were critical-point-dried using carbon dioxide. Root samples for observations of internal colonization were placed in dialysis tubing (MWCO 25,000) in 100% ethanol and frozen in liquid nitrogen. The latter samples were subsequently fractured on a copper disk (10 cm diam × 4 cm) in liquid nitrogen using a razor blade. Fractured root segments were placed in wells in the copper block, the block was removed from liquid nitrogen, the block (with wells filled with liquid nitrogen) was placed in a vacuum evaporator, and the evaporator was pumped down to 1 × 10 torr for 2 days. All dried root specimens were placed on mounting stubs, sputter-coated with gold-palladium to a thickness of 15 nm, and observed with a scanning electron microscope (International Scientific Instruments DS-130).

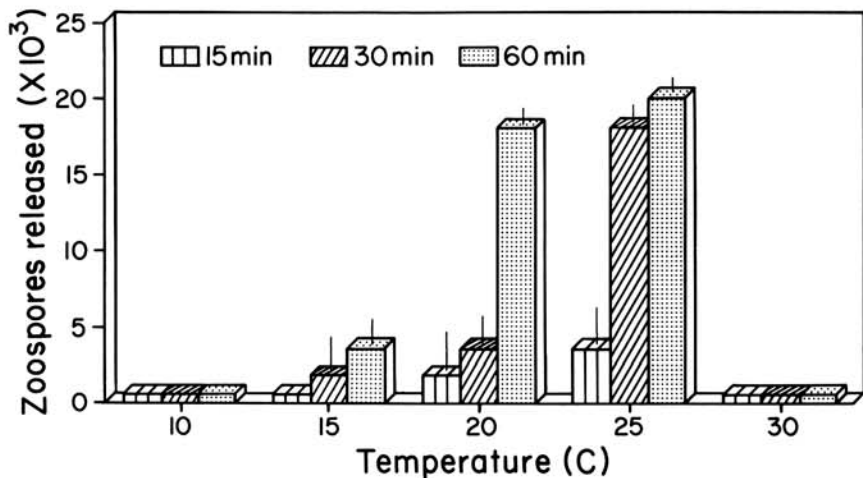
For bright-field microscopy, excised segments of roots were stained in acid



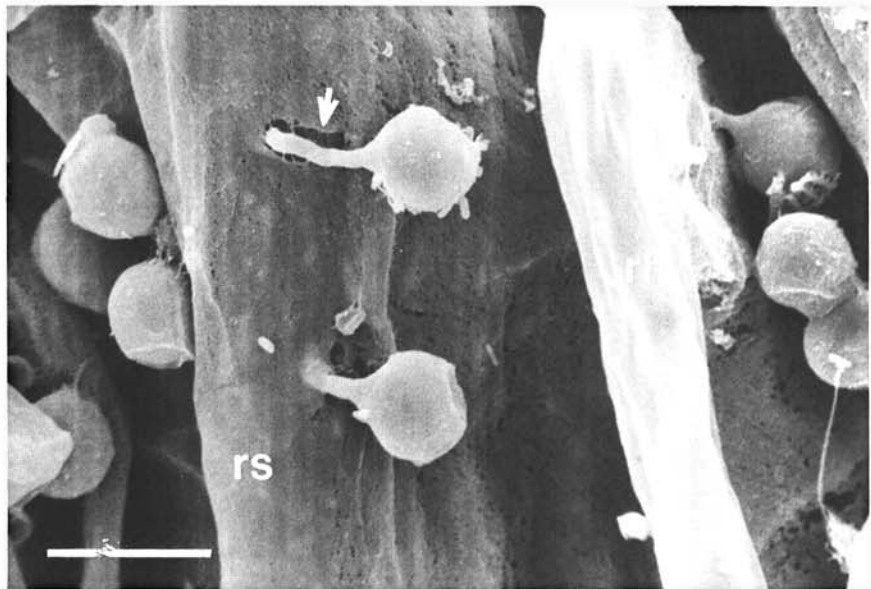
**Fig. 2.** Effect of root temperature and *Plasmopara lactucae-radicis* on shoot weight of lettuce cultured hydroponically. Four experiments, two at 18 C and two at 26 C, were done, each consisting of four plants per treatment; each treatment was replicated four times. Data were analyzed separately for each experiment by analysis of variance. Different letters within an experiment indicate significantly different mean weights between treatments at  $P = 0.01$ .



**Fig. 3.** Effect of temperature on sporangioophore production by *Plasmopara lactucae-radicis* on infected lettuce roots. Lines on bars represent standard error of the mean. Means are from two replicates in each of two trials.



**Fig. 4.** Effect of temperature on zoospore release from sporangia of *Plasmopara lactucae-radicis*. Lines on bars represent standard error of the mean. Means are from two replicates in each of two trials.



**Fig. 5.** Scanning electron micrograph of zoospore cyst germination and penetration of a lettuce root within 1 hr after inoculation with *Plasmopara lactucae-radicis*. Arrow indicates germination tube of a zoospore cyst on root surface (rs) next to a root hair (rh). Scale bar = 10 μm.

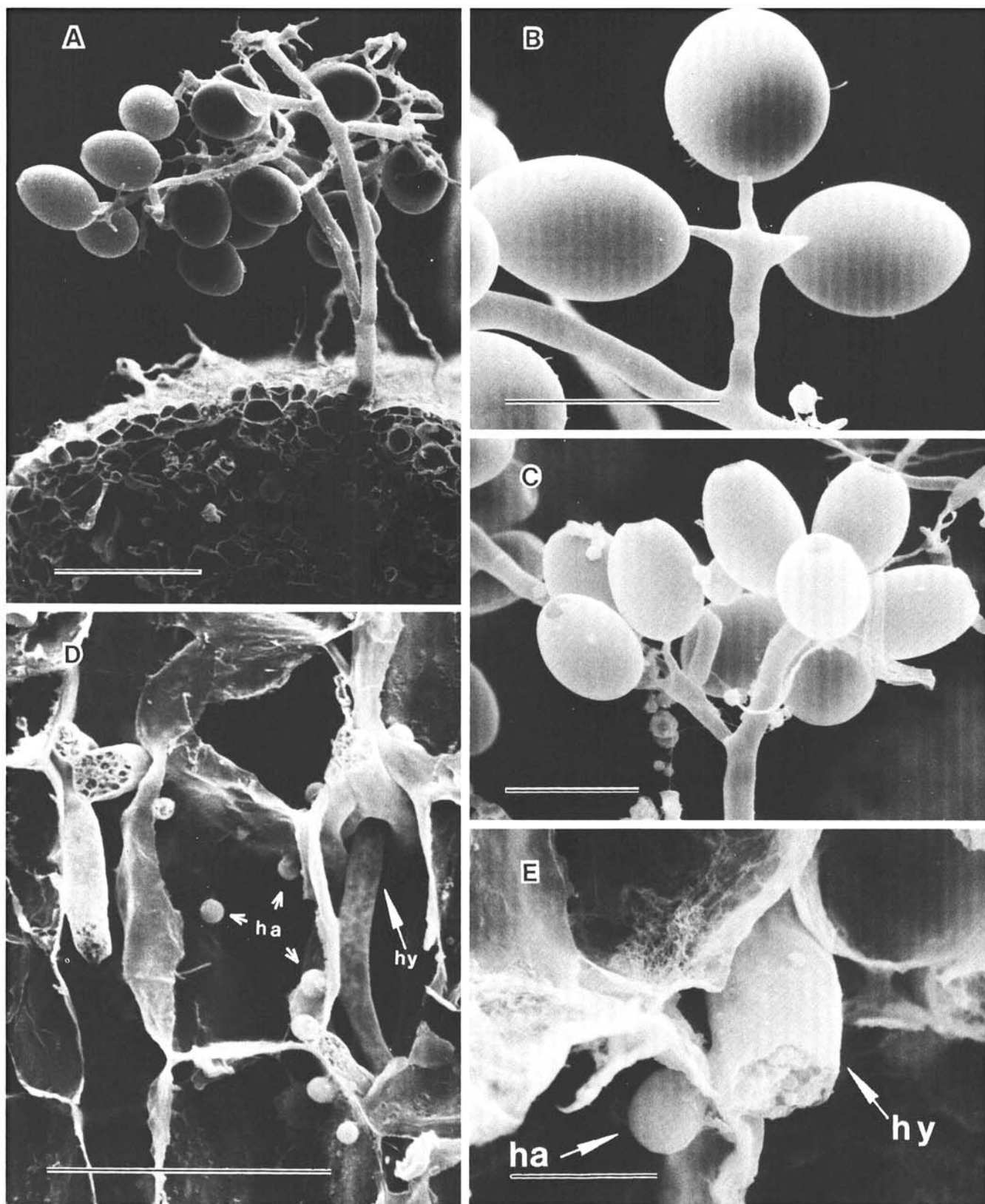
fuchsin, gently squashed, and examined under bright field and phase contrast.

**Cultivar susceptibility.** A plant infected with *P. lactucae-radici* was transplanted into a tub containing two 2-wk-old healthy lettuce plants of the

cultivars Sitonia and Salina. These two cultivars, in addition to Ostinata, are commonly used by commercial hydroponic growers. Portions of the root system of each cultivar were examined every 4 days over a 4-wk period as

described above for colonization by *P. lactucae-radici*. The experiment, conducted in the greenhouse at 26 C, was replicated twice and repeated three times.

**Cross-inoculation studies.** An isolate of *P. h. f. helianthi* and three susceptible



**Fig. 6.** Scanning electron micrographs of lettuce roots infected by *Plasmopara lactucae-radici*. (A) Cross section of root bearing a sporangiophore and sporangia. Scale bar = 100 μm. (B) Terminal sterigmata bearing sporangia. Scale bar = 50 μm. (C) Sporangia after zoospore discharge showing apical pore. Scale bar = 50 μm. (D,E) Intercellular hyphae (hy) and intracellular haustoria (ha) in the cortex of lettuce roots. Scale bars = 50 μm on D and 100 μm on E.

cultivars of sunflower (Krasnodarets, Interstate 7000, and 003) were used in cross-inoculation studies with *P. lactucae-radicis* and *Ostinata*. Leaves of sunflower and roots of lettuce bearing sporangia were placed separately in 9-cm-diameter petri plates containing 10 ml of sterile distilled water. Infested plates were then incubated at 20 C in order to induce zoospore discharge (20 C was chosen because *P. h. f. helianthi* did not produce zoospores at 25 C). Upon zoospore discharge (20 min for *P. lactucae-radicis* and 4 hr for *P. h. f. helianthi*), two 2-day-old seedlings of each cultivar, germinated on moist filter paper, were placed into each dish and incubated in the dark for 8 hr at 20 C. Inoculated seedlings were then removed, placed in growth pouches, and incubated at 100% relative humidity in a growth chamber at 22 and 24 C. Sporulation of the fungi on inoculated plants was recorded over a 3-wk incubation period.

## RESULTS

**Effect of temperature on infection.** *P. lactucae-radicis* caused significant reductions in fresh weights of shoots at 18 and 26 C (Fig. 2). Necrosis of portions of the root system was observed 10 and 14 days after inoculation at 26 and 18 C, respectively. Roots were not infected by *P. lactucae-radicis* at 12, 14, or 30 C.

**Effect of temperature on sporangiophore formation and zoospore release.** Sporangiophore formation on infected roots was initiated within 5–6 hr and maximum numbers of sporangiophores were formed on roots within 24 hr of incubation (Fig. 3). Both the rate of formation and the total numbers formed were greatest at 25 C (Fig. 3). Similarly, the rate of zoospore discharge and the total numbers of zoospores released from sporangia were greatest at 25 C (Fig. 4). Zoospore discharge from sporangia was initiated within 10 min and maximum numbers released were recorded within 60 min of incubation at all temperatures tested (Fig. 4).

**Cultivar susceptibility.** Roots of *Salina* were colonized by *P. lactucae-radicis* within 10 days after inoculation. At the termination of the experiment (28 days after inoculation), roots of *Salina* showed varying degrees of necrosis. No root necrosis or evidence of external colonization of roots of *Sitonia* by *P. lactucae-radicis* was detected despite extensive microscopic examination and continuous exposure of *Sitonia* to the fungus over the 4-wk period of cultivation.

**Effect of plant age on susceptibility.** Roots of 1- to 4-wk-old *Ostinata* plants were colonized by *P. lactucae-radicis* within 10 days of inoculation. Plant age did not appear to have an effect on colonization (i.e., degree of sporulation on the root surface or hyphae and haustoria within root tissue).

**Cross-inoculation studies.** *P. lactucae-radicis* and *P. h. f. helianthi* colonized only their respective hosts. Under the high humidity conditions of this study, sporulation of *P. lactucae-radicis* was observed on roots and cotyledons, but not on true leaves, of inoculated lettuce plants. Sporulation of *P. h. f. helianthi* was observed on roots, cotyledons, and true leaves of all inoculated sunflower cultivars.

**Penetration, colonization, and sporulation on roots.** The sequence of events involved in penetration, colonization, and sporulation of *P. lactucae-radicis* on roots of lettuce plants grown under hydroponic conditions at 26 C was as follows:

1. Zoospores were attracted (within seconds) primarily to the tip of roots, where they encysted and accumulated in large numbers.

2. Zoospore cyst germination and direct penetration of the root occurred within 1 hr after inoculation (Fig. 5).

3. Subsequent to penetration, a network of intercellular hyphae and laterally produced intracellular haustoria developed in the cortex of roots (Fig. 6 D,E). The number of haustoria produced ranged from three to 20 per root cell. Intercellular hyphae, which grew parallel to the long axis of the root, showed a directional growth toward the root tip and kept pace with root growth (Fig. 1F). Figure 7 shows the branching pattern of hyphae in roots. Hyphae were not observed in the hypocotyl or foliar portions of lettuce plants grown under greenhouse conditions.

4. Sporangiophores, originating from swollen vesicles immediately below the root epidermis (Fig. 6A), developed on the root surface in about 60 hr and produced mature zoosporangia (Fig. 6B,C) on primary terminal branches in about 62 hr after inoculation. Sporangiophores showed an indeterminate pattern of growth. Up to six consecutive sporangiophores (each originating from lower terminal sterigmata) have been observed.

5. Mature aplerotic oospores developed in cortical tissue of necrotic roots between 17 and 25 days after inoculation. Oospore germination was not observed.

## DISCUSSION

Results of our studies showed that *P. lactucae-radicis*, reported here for first time as a systemic root pathogen of lettuce, was responsible for significant reductions in yield in greenhouse pathogenicity tests. It is the only causal agent of downy mildew, other than *B. lactucae*, known on cultivated lettuce and is also unique for other reasons. First, the fungus is restricted to the root system, on which it sporulates profusely. The only other downy mildew pathogen known to infect and sporulate on the roots of its host is *P. h. f. helianthi*, the

causal agent of sunflower downy mildew. The latter fungus, however, also sporulates on leaves (2,3). No sporulation of *P. lactucae-radicis* was observed on true leaves of lettuce. Second, sporangiophores of *P. lactucae-radicis* (morphologically similar to those of *P. h. f. helianthi* on infected sunflower roots) show an indeterminate pattern of development, a morphological feature of the genus *Phytophthora*. The combination of indeterminate sporangiophore development, obligate parasitism, and restriction to the root system may position *P. lactucae-radicis* as a bridging organism between *Phytophthora* and higher fungi that cause downy mildew in the phylogenetic scheme proposed by Shaw (5).

The origin of *P. lactucae-radicis* is not known. The genus *Plasmopara*, however, is commonly found on many genera and species of the Compositae and is apparently native to North America (1). Presumably, the fungus occurs on a member of the Compositae in the vicinity of the commercial greenhouse. It is not surprising, given the reproductive capacity of the fungus (i.e., completion of its asexual life cycle in about 62 hr), that the fungus became widespread once it was introduced into the facility, which used a recirculating hydroponic system.

Temperatures of the nutrient solution at the onset of the epidemic in August 1987 ranged from 22 to 28 C. As shown in our study, temperatures of 20–26 C were optimal for pathogen and disease development. No disease occurred in pathogenicity tests at temperatures below 18 C, and the fungus was not detected on plants received from the commercial greenhouse during the winter production months when tem-

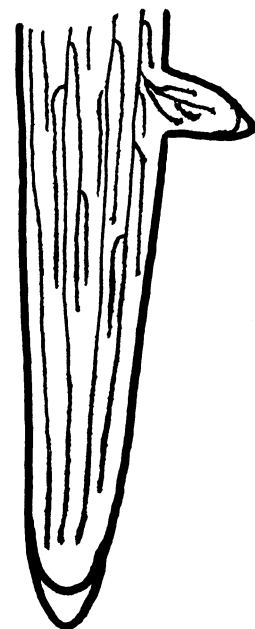


Fig. 7. Branching pattern of hyphae of *Plasmopara lactucae-radicis* in the cortex of a lettuce root.

peratures ranged from 9 to 16 C (*unpublished*). The fungus was again detected in May 1988 when temperatures rose to 23–26 C. Thus, the temperature range of the fungus, coupled with the use of the resistant cultivar Sitonia (resistance was confirmed by the cultivation of Sitonia in the commercial facility in September 1987), was integrated into a disease control strategy, i.e., the commercially preferred but susceptible cultivars Ostinata and Salina were grown during the winter production months and the resistant cultivar Sitonia was grown during the summer production months. Screening for additional cultivars resis-

tant to *P. lactucae-radicis* is currently in progress.

#### ACKNOWLEDGMENTS

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