Growth of *Penicillium oxalicum* as a Biological Seed Treatment on Pea Seed in Soil

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ABSTRACT

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When *Penicillium oxalicum*-treated seeds of *Pisum sativum* 'Little Marvel' were planted in either field or autoclaved soil, the conidia germinated, a hyphal network formed, and the antagonist sporulated on the seed coat by the third day after planting. Small dark lesions and some discoloration of cotyledons were noted after removal of seed coats from *P. oxalicum*-treated seeds planted in autoclaved soil, but lesions were superficial. Conidia of *P. oxalicum* were observed, either singly or in groups, on tap and secondary roots and root hairs of plants from *P. oxalicum*-treated seeds planted in either field or autoclaved soil. In field soil no germinated conidia of *P. oxalicum* were seen on roots, but in autoclaved soil, conidia germinated and hyphae of *P. oxalicum* grew between root hairs and on the root surface. Thus, conidia of *P. oxalicum* applied to seeds appeared to be active and the resulting mycelial network apparently functioned as a seed protectant, but conidia on root surfaces in field soil were apparently dormant.

Additional key words: spermosphere, rhizosphere, biological control.

The application of microorganisms to seeds to control seedborne and soilborne pathogens was reviewed (3,8,10). Antagonists applied to seeds not only have the potential of protecting the seed, but of being the initial colonizer of the root and having a protective effect against root-infecting pathogens. Harman et al (4) showed that when radish seeds coated with *Trichoderma hamatum* were incubated on moist filter paper for 2 days, *T. hamatum* grew on the seed coat. However, neither they (4) nor any other workers have shown that when organism-treated seeds are planted in the field, the antagonist becomes established (that is, that it grows, reproduces, or is active) in the spermosphere or the rhizosphere.

Previous work (7,15,16) showed that *Penicillium oxalicum* Currie and Thom was an effective seed treatment on pea, *Pisum sativum* L., in the greenhouse and field, and that it could be recovered from the rhizosphere (16,17).

The objectives of this study were to observe the activity of *P. oxalicum* on pea seeds and roots when *P. oxalicum*-treated seeds were planted in either field or autoclaved soil, as examined by light- and scanning electron microscopy. A brief report of this work has been given (14).

MATERIALS AND METHODS

Seeds of *Pisum sativum* 'Little Marvel' (a cultivar highly susceptible to soilborne pathogens of pea) were surface-treated in 5% NaOCl for 10 min, rinsed in sterile distilled water three times, and dried. Cultures of *Penicillium oxalicum* were grown on Difco Czapek-Dox agar (CDA) for 4–5 wk at 23 ± 2 C, and conidia were harvested by gently scraping the colony surface with a 1.5-cm camel's hair brush. Conidia were applied to seeds by adding 50 mg of dry conidia per 100 seeds in an Erlenmeyer flask shaken by hand 150 times to thoroughly coat the seeds. A 50-mg quantity of captan 80% WP (*N*-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide) per 100 seeds was applied as a dust in the same way. Nontreated seeds were shaken 150 times in a sterile flask. Harvesting of conidia, and disinfecting and treating seeds were done in a laminar-flow biological safety cabinet. Treated seeds were refrigerated at 5 C until planted.

In one experiment, *P. oxalicum*-treated and nontreated seeds were planted in storage dishes (8 cm deep by 10 cm in diameter) containing 200 cc of a mixture of vermiculite and soil (from a pea disease nursery) (1:9, v/v). Peas had been planted in the disease nursery every year for several decades and soilborne pea pathogens present included *Aphanomyces euteiches* Drechs., *Fusarium oxysporum* Schl. emend. Synd. and Hans. f. sp. *pisi*, *F. solani* (Mart.) App. and Wr. emend. Snyd. and Hans. f. sp. *pisi*, *Pythium* spp., and *Rhizoctonia solani* Kühn. Seeds that had received the same treatments also were planted in soil (from the same source) that had been autoclaved 1 hr per day, 2 days in succession. Ten seeds were planted per container at 1.5–2.0 cm depth; containers were covered with glass lids, and placed in an incubator at 21 C, 12 hr light (5,500 lux) and 12 hr dark. There were three replicates of autoclaved soil and field soil for both *P. oxalicum*-treated and nontreated seeds. In the second experiment, three replicates of *P. oxalicum*-, captan-, and nontreated seeds were planted in autoclaved soil in covered deep storage dishes and incubated at 21 C, as described for the previous experiment. Also, *P. oxalicum*, captan-, and nontreated seeds (15 seeds per row) were planted (2.5-cm depth) in each of three metal flats (36 × 25 × 9 cm) containing 2 L of soil from the pea disease nursery, and incubated in a greenhouse at 21 ± 4 C. Soil had been mixed as previously described and was watered daily or every second day.

In both experiments, seedlings were gently removed from the soil with a forceps, seeds were immediately severed from roots with a razor blade, and then seeds and roots were placed in separate petri dishes or on paper towels. Excess soil was removed by gently tapping the seed or root on a paper towel, although not all soil was removed. The seed coat was then nicked with a razor blade, pulled away from the seed with a forceps, and cut into pieces approximately 2–4 mm^2. Epidermis was peeled from the root (about 1–3 cm of tap root proximal to the point of seed attachment) or else the root (tap or secondary) was cut into segments 1.0–1.5 cm long and then cut longitudinally with a razor blade. Specimens were placed on clean glass slides and stained with a 1% solution of phloxine in water for 10–30 sec; 1–2 drops of lactophenol was added to the specimen or else the specimen was transferred to a drop of lactophenol on another slide. Several seed coats and roots were examined with a Nikon light microscope at 2 or 3 days after

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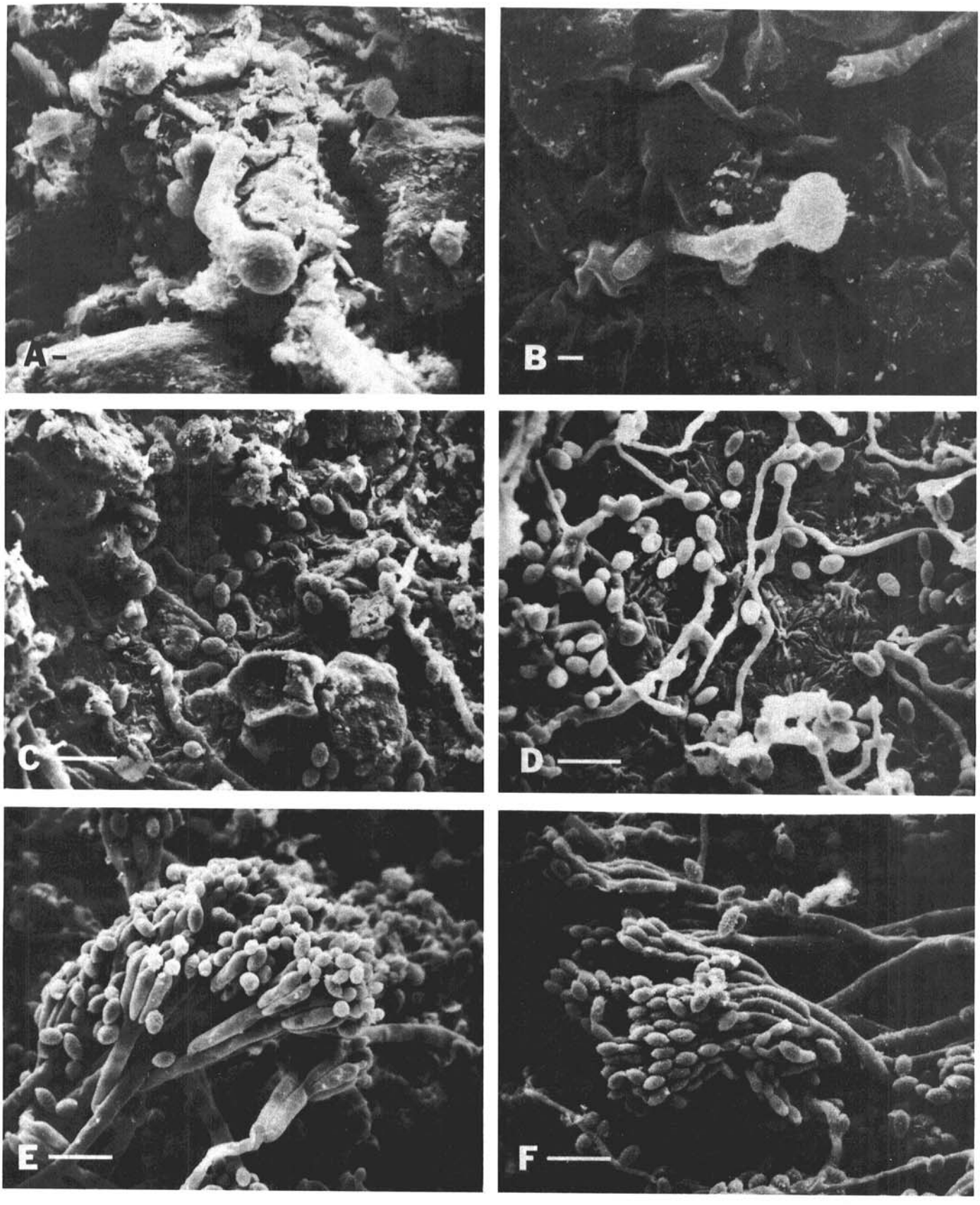

Fig. 1. Electron micrographs of seed coats of *Pisum sativum* 'Little Marvel' 5 days after planting showing germination of *Penicillium oxalicum* conidia when *P. oxalicum*-treated seeds were planted in **A,** field soil and **B,** autoclaved soil; formation of a network of hyphae of *P. oxalicum* when *P. oxalicum*-treated seeds were planted in **C,** field soil and **D,** autoclaved soil; and sporulation of *P. oxalicum* when *P. oxalicum*-treated seeds were planted in **E,** field soil and **F,** autoclaved soil. Scale bars on micrographs A and B equal 1 μm and scale bars on micrographs C–F equal 10 μm.

planting, and then every 2–3 days for 1.5–2.0 wk after planting. Photographs were taken with a Nikon Microflex "EFM" semi-automatic photomicrographic attachment (Nippon Kogaku Inc., 623 Stewart Avenue, Garden City, NY 11530).

Seed coats and roots of *P. oxalicum*-, captan-, and nontreated seeds were collected 5 days after planting from pea field soil and from autoclaved soil, and prepared for examination by scanning electron microscopy (SEM). Pieces of seed coat approximately 2 mm^2, and root pieces approximately 2–3 mm long and split longitudinally, were fixed for 12 hr in 2.5% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0). Specimens were postfixed in 0.01 M phosphate-buffered (pH 7.0) 2% osmium tetroxide for 1 hr, dehydrated in a graded ethanol series and dried in a Bomar SPC-50 EX critical-point drier (The Bomar Co., Tacoma, WA). Three segments of root and six pieces of seed coat per treatment (three showing the surface of the seed coat and three showing the inner surface of the seed coat) were mounted on SEM specimen stubs with Carbon DAG (Structure Probe, Inc., West Chester, PA) and coated with platinum/palladium in a vacuum evaporator. Root and seed coat specimen stubs were prepared for each seed treatment collected from pea field soil and from autoclaved soil. Specimens were examined in a Phillips 500 scanning electron microscope (Phillips, Eindhoven, The Netherlands) and photographs were taken with Polaroid PN/55 film.

To determine the effect of *P. oxalicum* on cotyledons, seed coats were removed from several seeds 1–2 wk after planting them in autoclaved soil, and cotyledons were sectioned with an International microtome cryostat. The sections were stained as previously described for seed coat and root samples, and examined by light microscopy. Also, superficial lesions were removed from the seed with a razor blade, and examined by light microscopy.

RESULTS

Observations with the light microscope on activity of *Penicillium oxalicum* on seeds planted in soil collected from the pea disease nursery and in autoclaved soil were corroborated by SEM. Electron micrographs taken of seeds and roots removed from these soils 5 days after planting are usually shown in preference to light microscope photographs in the figures of this report. However, the micrographs are representative of events observed with the light microscope.

Seed coat. Two days after *P. oxalicum*-treated seeds had been planted in either field or autoclaved soil, conidia of *P. oxalicum* had germinated on the ridges and in the valleys of seed coats (Fig. 1A and B). Although not all conidia had germinated, a network of hyphae was visible on the seed coat (Fig. 1C and D). Soil particles tended to cling to the surface of *P. oxalicum*-treated seeds planted in field soil, but this did not occur on seeds planted in autoclaved soil (Fig. 1A to D). At 3 days after planting, *P. oxalicum* had sporulated on some *P. oxalicum*-treated seeds in both field or autoclaved soil (Fig. 1E and F).

Fungi and bacteria were not seen on captan- or nontreated seeds planted in autoclaved soil. The seed coats of captan-treated seeds planted in field soil appeared to be free from fungi, but bacteria were present on the seed coat; the seeds also were relatively free from adhering soil. Seed coats of nontreated seeds planted in field soil were colonized by bacteria and by fungi, and soil particles tended to adhere to the seed.

Mycelia of *P. oxalicum* were not seen in SEM micrographs of the inner surface of seed coats of *P. oxalicum*-treated seed planted in autoclaved soil for 5 days, but they were observed with a light microscope on samples collected 7 days after planting. Mycelia were observed on the inner surface of seed coats of *P. oxalicum*-treated and nontreated seeds planted in field soil, but the fungi could not be identified by the mycelium alone.

Cotyledon surface. After seed coats had been removed from *P. oxalicum*-treated seeds planted in autoclaved soil, small dark lesions and some discoloration of cotyledons were visible beginning approximately 6 days after planting (Fig. 2). Cotyledons of captan- or nontreated seeds planted in autoclaved soil were not discolored. If planted in field soil, seeds that had been either treated or not treated with *P. oxalicum* had discolored cotyledons; cotyledons of seeds treated with captan were not discolored.

Cotyledon lesions on *P. oxalicum*-treated seeds planted in autoclaved soil involved from one to many cells, and were circular or irregular shaped (Fig. 3A). Intercellular darkening of cells between adjacent small lesions occurred, probably because the lesions were increasing in size (Fig. 3B). In cross sections made 14 days after the seeds had been planted the lesions appeared to be superficial (Fig. 3C). Cross sections of cotyledons of nontreated seeds planted in autoclaved soil revealed only healthy tissues (Fig. 3D).

Roots. Conidia of *P. oxalicum* were found singly and in groups on primary and secondary roots and root hairs of plants from *P. oxalicum*-treated seeds planted in either field or autoclaved soil (Fig. 4). However, in field soil, no conidia of *P. oxalicum* on roots had germinated within 1.5–2.0 wk after planting. *P. oxalicum* conidia were usually associated with soil particles or amorphous material clinging to roots and these materials could obscure the germinated conidia. Conidia were not observed on roots of plants from captan-treated or nontreated seeds planted in field soil, so it was assumed that conidia observed on roots of plants from *P. oxalicum*-treated seeds were conidia of *P. oxalicum*. It is not known how the conidia entered the rhizosphere.

In field soil, unidentified fungi grew on the root surface and some hyphae penetrated the roots of plants from treated and nontreated seeds. There were bacteria on the root surface and a delicate weblike material, probably mucigel, on the root and root hairs.

When *P. oxalicum*-treated seeds were planted in autoclaved soil, mycelium of *P. oxalicum* grew on the root surface (Fig. 5A) and

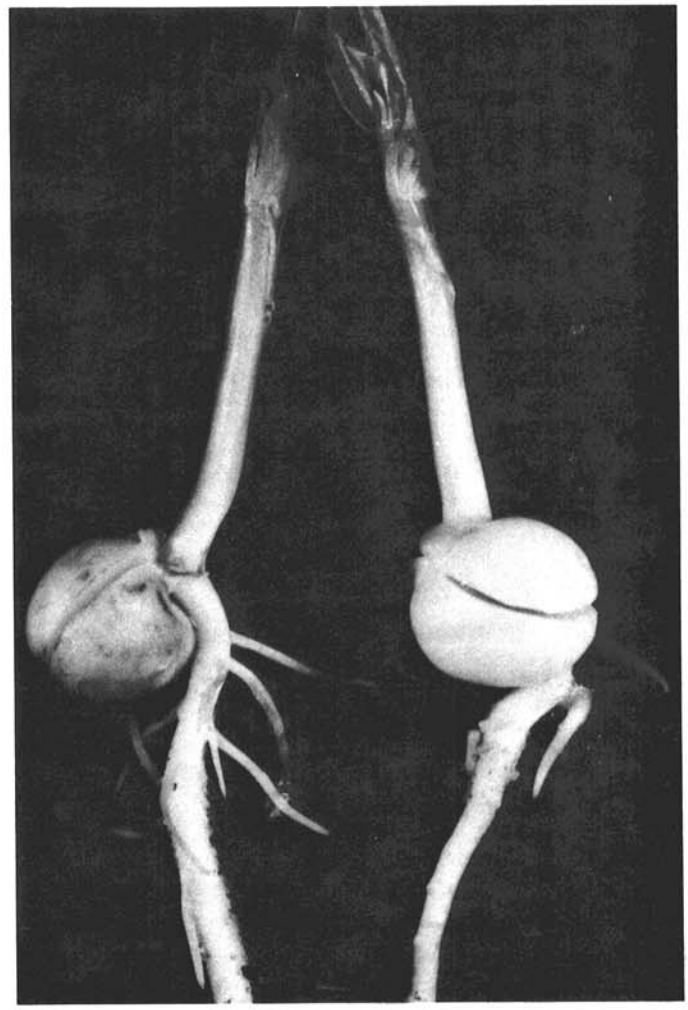

Fig. 2. Seedlings of *Pisum sativum* 'Little Marvel' after seed coats were removed from a *Penicillium oxalicum*-treated seed (left) and a nontreated seed (right) 10 days after planting in autoclaved soil. Note the discoloration on cotyledons of the *P. oxalicum*-treated seed.

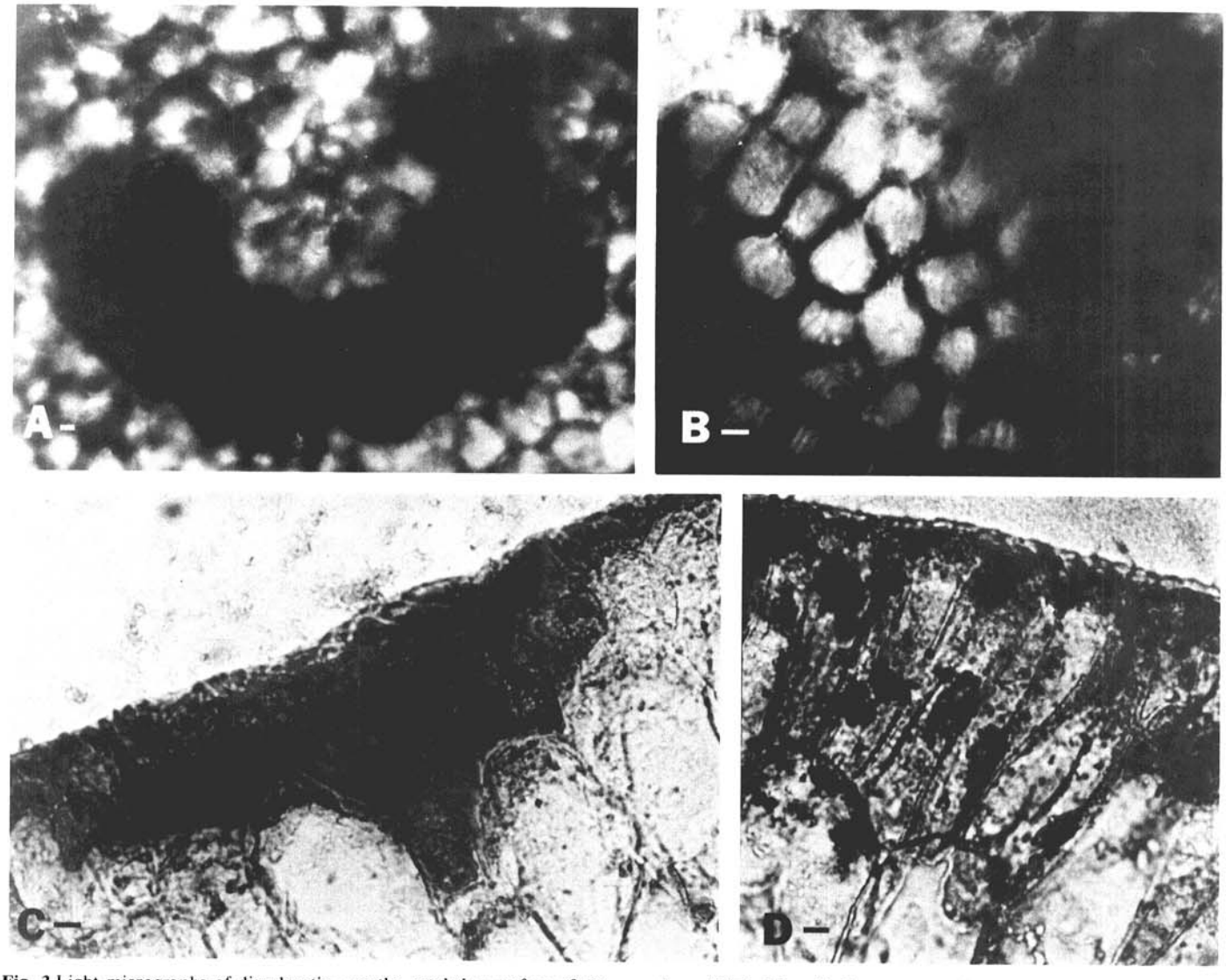

Fig. 3. Light micrographs of discoloration on the cotyledon surface of *Pisum sativum* 'Little Marvel' after removal of the seed coat of a *Penicillium oxalicum*-treated seed showing **A,** a lesion involving several cells, **B,** intercellular darkening between lesions, and **C,** a cross section of a *P. oxalicum* lesion compared to **D,** a cross section of an unblemished cotyledon 14 days after seeds were planted in autoclaved soil. The scale bar in each micrograph equals 10 μm.

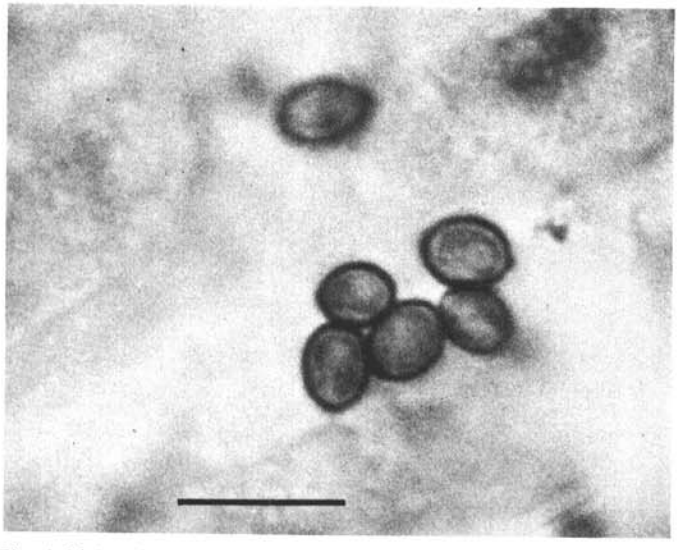

Fig. 4. Light micrograph of conidia of *Penicillium oxalicum* on roots of *Pisum sativum* 'Little Marvel' 2 days after planting *P. oxalicum*-treated seeds in field soil. The scale bar equals 10 μm.

between root hairs of seedlings (Fig. 5B). With SEM observation of roots grown in autoclaved soil, mycelium of *P. oxalicum* could not be traced back to conidia because of soil particles adhering to the roots. By using light microscopy, *P. oxalicum* mycelium could readily be traced back to conidia, although not all conidia on the roots had germinated.

DISCUSSION

The growth of *P. oxalicum* on the seed coat within 48 hr of planting infested seeds in field soil explains why seed treatment with *P. oxalicum* protected seedlings against preemergence damping-off (7,15). Exudates from germinating pea seeds would provide a readily available source of carbohydrates (9) for overcoming fungistasis, and allow the antagonist to grow. Short and Lacy (12) found that the greatest amount of carbohydrate exuded from pea seed occurred during the first 18 hr of incubation at 22 or 30 C, and the experiments in this paper were done at 21 C. *Penicillium oxalicum* covered the seed coat and could protect the seed physically in addition to reducing the amount of seed exudates available to pathogens. These mechanisms were also suggested for *T. hamatum* applied to radish seed (4).

Although *P. oxalicum* was a beneficial seed protectant in field soil when compared to nontreated seed (15–17), it formed

superficial lesions on the cotyledons in both field and autoclaved soil (Figs. 2 and 3). Similar lesions occurred on cotyledons of established seedlings of nontreated seeds germinating in field soil, and were observed by others (1). However, these lesions may represent normal colonization of senescent tissue as the seedling develops. Reserves began to move from the cotyledons at 5–8 days after planting (1) and the greatest loss of reserve material from the cotyledons occurred 9–21 days after planting, although cotyledons alone did not support maximum growth of the seedling (2). Mycelium of *P. oxalicum* was observed on the inner surface of the seed coat, which suggests that *P. oxalicum* could have entered the cotyledon, or that cells of the cotyledon were killed prior to penetration. Johann et al (5) found that on some lines of inbred and hybrid dent corn, *P. oxalicum* produced oxalic acid, which killed seedling tissue in advance of penetration.

The apparent failure of *P. oxalicum* to become established in the rhizosphere may account for the lack of control of postemergence damping-off and root rot by seed treatment (13,15). Also, the presence of conidia of *P. oxalicum* in the rhizosphere did not affect populations of actinomycetes and other bacteria, *F. oxysporum*, *F. solani*, and the total number of fungi (except for a greater population of other *Penicillium* species) compared to populations of these organisms in the rhizosphere from captan- or nontreated seeds (17). This observation is similar to that of Merriman et al (11) who reported that *Bacillus subtilis* and *Streptomyces griseus* were not effective colonizers of the rhizosphere when they were used as seed inoculants. However, inoculation of peach seed with *Agrobacterium radiobacter* var. *radiobacter* strain 84 to control crown gall resulted in its predominance on roots, underground stems, and in soil around plant crowns (6).

In conclusion, conidia of *P. oxalicum* applied to seeds were active and established on the seed coat shortly after seeds were planted, but no evidence was found for activity of conidia on roots in field soil. This suggests that *P. oxalicum* acts primarily as a seed protectant.

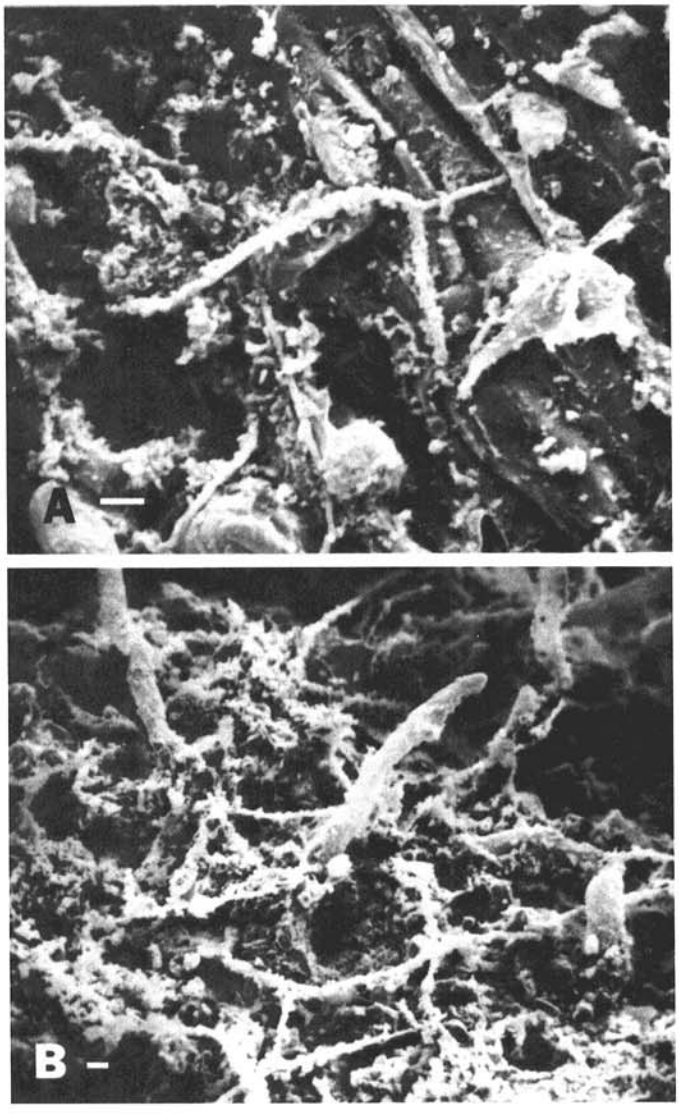

Fig. 5. Electron micrographs showing mycelium of *Penicillium oxalicum* **A,** on the root surface and **B,** around a root hair 5 days after *P. oxalicum*-treated seeds of *Pisum sativum* 'Little Marvel' were planted in autoclaved soil. Scale bars on micrograph equal 10 μm.

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