

## Factors Affecting *Penicillium oxalicum* as a Seed Protectant Against Seedling Blight of Pea

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### ABSTRACT

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Effectiveness of *Penicillium oxalicum* as a pea seed treatment was improved significantly by: (i) using spores harvested from a Czapek-Dox or a potato-dextrose agar medium and to a lesser extent from an oat grain medium; (ii) applying at least  $3 \times 10^6$  and preferably  $6 \times 10^6$  spores per seed; and (iii) storing organism-coated seeds for 2 mo at 5 C

instead of 24 C prior to planting. Spore ages from 20 to 125 days were equally effective when applied as inoculum for seed treatment. *Penicillium oxalicum* was not deleterious to pea germination or otherwise pathogenic to peas. *Penicillium oxalicum* was more effective in controlling preemergence than postemergence damping-off.

*Additional key words:* biological control.

In a previous study (3) 100 isolates of microorganisms were used to coat seeds of peas in an attempt to increase stand establishment and reduce root rot. Among these, seed treatment with a culture of *Penicillium* usually resulted in more seed germination and less root rot compared to plants that received no seed treatment. This original culture comprised four *Penicillium* spp. One of these species, *P. oxalicum*, increased percentage germination and reduced root rot severity, and it was nearly as effective as captan in protecting seedlings. We decided not to continue searching for a more effective organism but to improve the performance of this one (7).

Experiments were designed to study: (i) the effect of *P. oxalicum* on seeds planted in steamed and nonsteamed soil; (ii) the importance of the substrate on which inoculum of the antagonist was grown; (iii) the number of spores needed on pea seeds to protect seeds against disease; (iv) the effect of storage temperature on treated seeds; and (v) the importance of spore age of the antagonist.

### MATERIALS AND METHODS

*Penicillium oxalicum* Currie & Thom was grown on Difco Czapek-Dox agar (CDA) or on Difco potato-dextrose agar (PDA). Spores were harvested dry by gentle scraping of the agar surface with a blunt-ended spatula or a bent glass rod. Spores were applied to seeds as a dust by mixing 100 mg of spores with about 200 seeds of *Pisum sativum* L. 'Little Marvel' (a cultivar known to be highly susceptible to common root pathogens) in a 125-ml Erlenmeyer flask which then was shaken by hand

150 times. Seeds then were coated thoroughly even though some spores remained in the flask. Spores were distributed in the depressions of the seed but were more concentrated on the ridges. Captan 80% WP (N-trichloromethyl-mercapto-4-cyclohexene-1, 2-dicarboximide) was applied as a dust in the same way and the excess was screened off. The nontreated seeds also were shaken 150 times in a sterile flask. Spore harvesting and seed treatment procedures were done in a laminar-flow biological safety cabinet.

Seeds were planted in the greenhouse in soil collected from a pea "disease nursery" in which peas had been planted for several decades. Pea root pathogens present in the soil included *Aphanomyces euteiches* Brechs., *Fusarium oxysporum* Schl. emend. Snyd. & Hans. f. sp. *pisi*, *F. solani* (Mart.) App. & Wr. emend. Snyd. & Hans. f. sp. *pisi*, *Pythium* spp., and *Rhizoctonia solani* Kühn. The soil was screened, mixed with vermiculite [nine parts soil to one part vermiculite (v/v)] and then 10 liters were put into a metal flat (50 × 35 × 10 cm) in a greenhouse at 21 ± 4 C. Twenty-five seeds were planted (2.5 cm deep) per treatment for each of the eight replicates in a randomized block design, unless otherwise indicated. After the seeds were planted, sand was sprinkled over the soil surface in each flat.

Seedling emergence was recorded every 1-3 days for 2 wk after planting, and thereafter every few days until plants were harvested. Four wk after planting, pea plants were removed from the soil, washed, weighed, and rated for root rot. The severity of root rot was evaluated on a scale of 0-4, where 0 = plants with a slight amount of water-soaking or light brown discoloration on the roots but with good primary and secondary root development (no plants were entirely free from symptoms); 1 = plants with brown discoloration but not involving the entire root system, tissue firm and with good primary or secondary

root development; 2 = plants with water-soaking and browning of roots and epicotyl, tissue soft but not collapsed and with some primary or secondary root development; 3 = as above, but with more extensive browning and the cortex easily removable, also including plants with roots or shoots only; and 4 = seeds that did not germinate. The number of plants in each rating class was multiplied by its class number; values were totaled and the sum was divided by the number of seeds planted.

To estimate the number of spores of *P. oxalicum* per seed, spores were removed from seeds by placing a pea seed in a vial with 5 ml of 2% sodium dodecyl sulfate. The vial was placed in a water bath at 100 C for 10 min to free spores from the seed coat and then the vial was placed in an ultrasonic vibrator for 7-8 min to break up clumps of spores. Spores were counted with a Neubauer hemacytometer; the volume of the remaining spore suspension was measured because the seed had imbibed water. Four or five seeds were used per treatment.

To estimate spore viability, 5 or 10 mg of spores were placed in a Waring Blendor together with 100 ml of potato-dextrose broth (PDB) and two drops of dilute Tween-20 (one drop of Tween-20 concentrate in 10 ml of PDB). The Blendor was run at low speed for 30 sec. Spore counts in the broth were determined with a Neubauer hemacytometer. The 100 ml of broth were then serially diluted in PDB until an appropriate spore concentration (to attain 20-40 colonies/petri dish) was reached and then a 1-ml aliquot was pipetted onto a petri dish of PDA. Three dishes were prepared for each dilution and incubated at 24 C for 3-4 days, when colonies were counted.

**Steamed versus nonsteamed soil.**—Pea seeds were added to 21-day-old cultures of *P. oxalicum* grown on PDA and shaken by hand 50 times and seeds were coated thoroughly with spores. Captan was applied as a dust in the same way and nontreated seeds were shaken in a sterile petri dish. Ten seeds of each treatment were then planted in a 360-ml paper cup containing either steamed (100 C for 2 hr) or nonsteamed field soil and kept at 24 C.

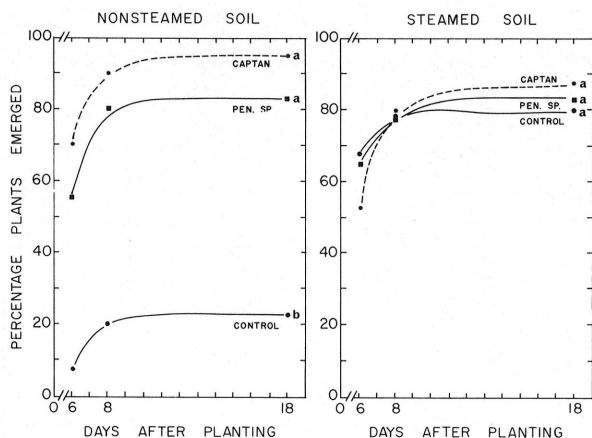


Fig. 1. Percentage emergence of plants from captan-, organism-, and nontreated pea seeds planted in nonsteamed and steamed soil. Final emergence means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .

There were four replicates per treatment.

**Substrate.**—Spores of *P. oxalicum* were harvested from 48-day-old cultures grown on CDA or PDA and from 49-day-old cultures grown on sterile oats. Oat grains and water (30% by weight) were added to 250-ml Erlenmeyer flasks and autoclaved at 121 C for 1 hr on each of 3 successive days; then this medium was inoculated with *P. oxalicum* and incubated at 24 C. To harvest spores from oats, the oats were placed in a sieve with a 1.18-mm screen and shaken vigorously. Since some debris from the oat grains also was collected, spores were again sieved through a 0.5-mm screen. Nontreated pea seeds also were (i) placed directly on oat grains on which *P. oxalicum* was sporulating and (ii) placed on sterile oat grains; in both treatments 20 cc of oat grains were applied per 33-cm row in a flat.

**Amount of spore inoculum on seeds.**—Spores of *P. oxalicum* were harvested from 49-day-old cultures grown on CDA, and 5, 10, 50, 100, or 150 mg per dosage were added to approximately 200 pea seeds.

**Age of spores.**—Spores of *P. oxalicum* were harvested from cultures 16, 37, 50, and 98 days old grown on CDA, placed in 7-ml vials and kept at 24 C for 8-10 days. Spores from the older cultures were drier than spores from younger cultures; to insure uniform moisture content of the spores, all vials were covered with cotton plugs and placed in a desiccator for 2 wk. Spores were harvested from an additional set of 9-day-old cultures and placed in a desiccator for 8 days. Vials were shaken occasionally during storage in the desiccator to redistribute spores. After seeds were treated they were stored in a refrigerator at 5 C for 3 days; at planting time, the ages of spores on seeds were 20, 41, 62, 75, and 125 days old.

Even though we tried to keep the number of spores per

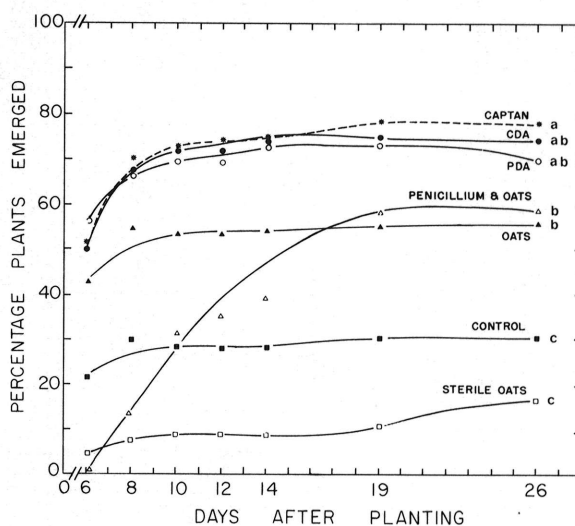


Fig. 2. Percentage emergence of plants from pea seeds coated with captan or with spores of *Penicillium oxalicum* from Czapek-Dox agar, potato-dextrose agar, or sterile oats; percentage emergence from pea seeds planted with (i) oat grains with sporulating *P. oxalicum*, (ii) sterile oat grains, or (iii) a nontreated control in a greenhouse at  $21 \pm 4$  C. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .

seed as uniform as possible for each spore age, still there was variation among treatments. Spores 20, 41, 62, 75, and 125 days old averaged 8.4, 9, 9.8, 8.2, and  $5.7 \times 10^6$  spores per seed, respectively. About 100% of the spores 20, 41, and 72 days old were viable compared to about 70% of the spores 101 and 125 days old.

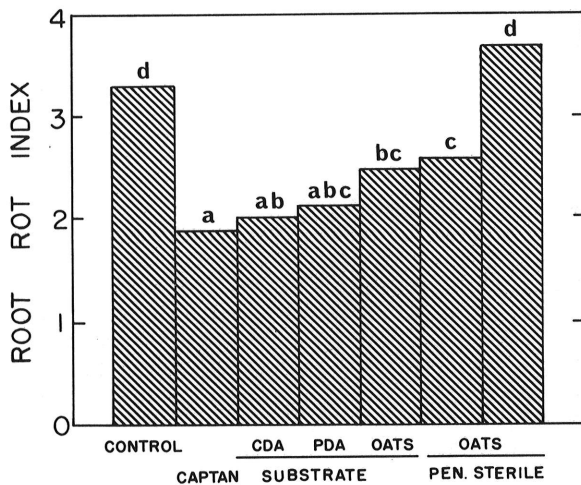
**Storage of treated seeds at 5 and 24 C.**—Spores were harvested from 48-day-old cultures grown on CDA and PDA. After seeds were treated, half of the seed lot was stored in a refrigerator at 5 C and half was stored at 24 C

for 2 mo, and then seeds were planted in the greenhouse. The experimental design was a randomized block with 20 seeds per treatment for each of 10 replicates per flat.

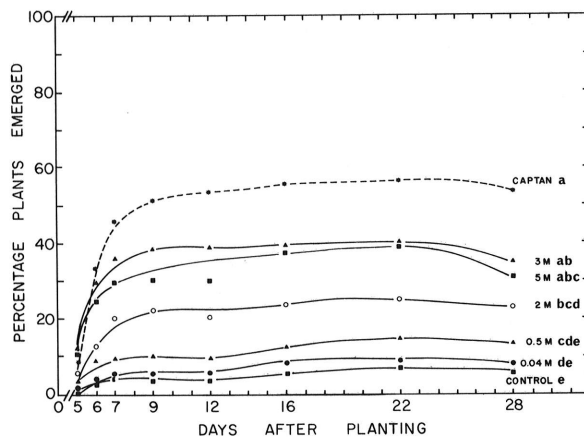
Analyses of variance were performed and means were compared by Duncan's new multiple-range test ( $P = 0.01$ ). For emergence and fresh weight determinations arc sine and square root transformations, respectively, were done prior to analysis of variance, but means presented in this paper have been transformed back to the original scale (6).

**RESULTS**

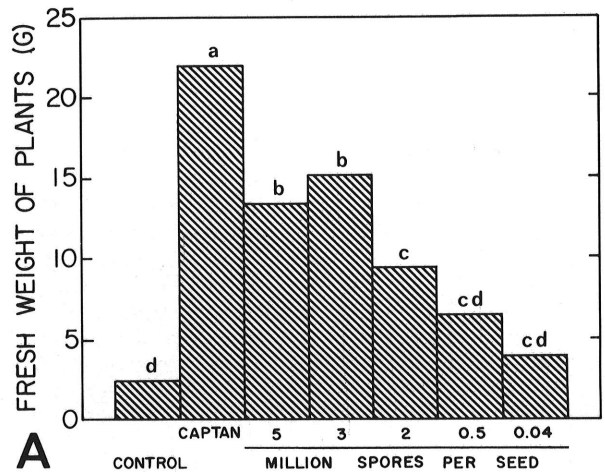
**Effect of treated seed in steamed soil.**—Of seeds planted in nonsteamed soil those coated with *P. oxalicum*



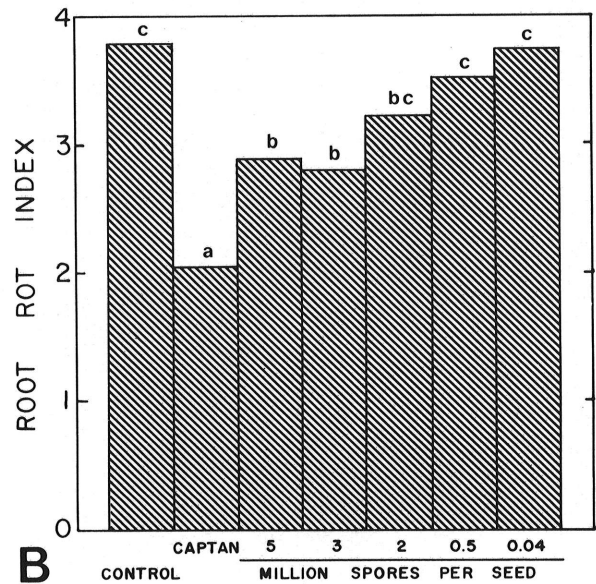
**Fig. 3.** Root rot index (range 0-4) of plants from pea seeds coated with captan or spores of *Penicillium oxalicum* grown on three substrates, compared to the root rot index from pea seeds planted with (i) *P. oxalicum* sporulating on oat grains, (ii) sterile oat grains, and (iii) a nontreated control in a greenhouse at  $21 \pm 4$  C, 25 days after planting. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .



**Fig. 4.** Percentage emergence of plants from pea seeds coated with captan or different amounts of spores of *Penicillium oxalicum* compared to percentage emergence from nontreated seeds planted in soil from the pea disease nursery in a greenhouse at  $21 \pm 4$  C. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .



**A**



**B**

**Fig. 5-(A, B).** Effects of preplant treatment of pea seed with spores of *Penicillium oxalicum*. **A)** Fresh weight and **B)** root rot index (range 0-4) of 28-day-old plants from pea seeds coated with captan or different amounts of spores of *P. oxalicum* compared to plants from nontreated seeds planted in soil from the pea disease nursery in a greenhouse at  $21 \pm 4$  C. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .

or captan resulted in significantly higher rates of emergence than those not treated (Fig. 1). There were no differences in emergence between organism-, captan-, and nontreated seeds planted in steamed soil, indicating that *P. oxalicum* had no detrimental effect on seed germination.

**Effect of substrate on antagonist's performance on seed.**—All seed treatments gave significantly better rates of emergence than did the nontreated control or pea seeds planted with sterile oat grains. Seedling emergence from seeds treated with *P. oxalicum* grown on CDA or PDA was as good as emergence from seeds treated with captan (Fig. 2). All three of these treatments were superior to *P. oxalicum* from oat grains (Fig. 2). Emergence was delayed when seeds were planted with oat grains on which *P. oxalicum* was sporulating, but by about 19 days after planting the emergence of seedlings equalled that from seeds coated with spores separated from oat grains. The same trends were observed in the root rot index (Fig. 3). The high index value given for the nontreated control reflects the high concentration of pathogens in the soil. Sterile oat grains had a higher moisture content than oat grains colonized by *P. oxalicum* and may have served as a substrate for the pathogens.

By weighing the seeds before and after coating them and by determining the number of spores per unit weight of inoculum, about  $6 \times 10^6$  spores were applied per seed for each substrate. About 100% of the spores harvested from PDA and CDA and about 26% of the spores harvested from oats were viable.

**Effect of number of spores per seed.**—Of about 200 seeds coated with 5, 10, 50, 100, or 150 mg of inoculum of *P. oxalicum*, the average number of spores per seed was 0.04, 0.5, 2, 3, and  $5 \times 10^6$  spores, respectively. When 100 or 150 mg of spores were applied, seeds were covered thoroughly and there were some spores left over. For the

lower amounts of inoculum, there were no excess spores remaining in the flask after seeds were treated; at 5 mg of inoculum, spores were not readily apparent on the seed coat. Spore viability was 100%.

A significantly greater rate of seedling emergence was obtained from seeds treated with captan and with  $3$  or  $5 \times 10^6$  spores per seed, than for nontreated seeds and seeds

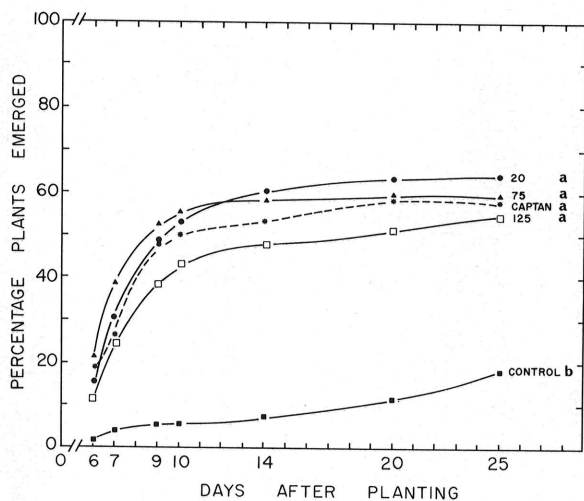
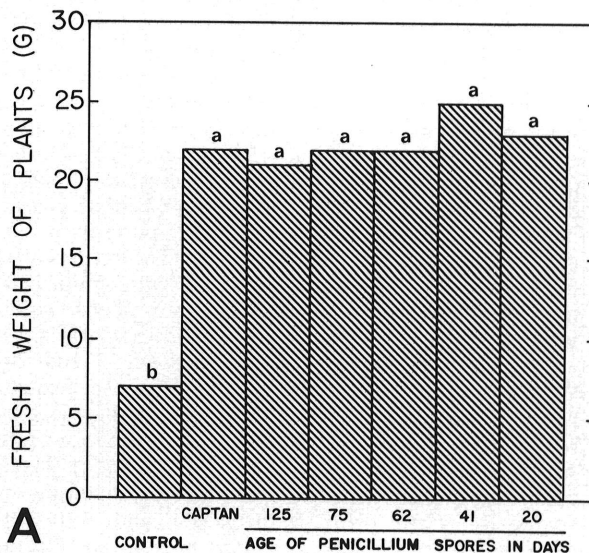
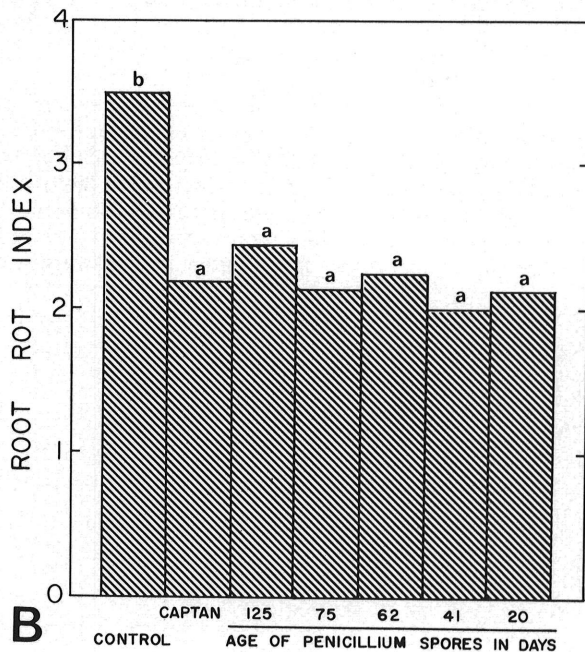


Fig. 6. Percentage emergence of plants from pea seeds coated with captan or spores of *Penicillium oxalicum* from cultures 20, 75, and 125 days old, compared to percentage emergence of plants from nontreated seeds planted in soil from the pea disease nursery in a greenhouse at  $21 \pm 4$  C. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .



A



B

Fig. 7-(A, B). Effects of age of inoculum of *Penicillium oxalicum* as a seed treatment. A) Fresh weight and B) root rot index (range 0-4 of 25-day-old pea plants grown from seeds coated with captan or spores of *P. oxalicum* from cultures 20, 41, 62, 75, and 125 days old compared to plants from nontreated seeds planted in soil from the pea disease nursery in a greenhouse at  $21 \pm 4$  C. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .

treated with 0.04, 0.5, and  $2 \times 10^6$  spores per seed (Fig. 4). The extremely low rate of emergence in the nontreated control was correlated with a high incidence of disease. The soil had been planted to peas twice previously in the greenhouse, which may explain why the emergence rate of captan-treated seed, the best seed treatment, was only 53%. About 3 wk after planting, some postemergence damping-off occurred, which suggested that the effect of coating seeds with captan or spores of *P. oxalicum* was only temporary. Fresh weight and root rot ratings gave results similar to those for emergence (Fig. 5).

**Effect of spore age.**—Seeds coated with spores of *P. oxalicum* of different ages emerged at the same rate as seeds coated with captan; again, significantly greater emergence rates were obtained for treated than for nontreated seeds (Fig. 6). Values for emergence of seeds treated with spores 41 and 62 days old were intermediate between the values of seeds treated with spores 20 or 125 days old, and are not included in Fig. 6. The fresh weight of plants and the root rot index (Fig. 7) also confirmed that organism- and captan-treated seeds gave significantly better results than did nontreated seeds.

**The effect of storing treated seeds at 5 and 24 C.**—Seedling emergence was less for organism-treated and nontreated seeds stored at 24 C compared with seeds stored at 5 C (Fig. 8). No decrease in emergence occurred with captan-treated seeds. Storage of seeds at 24 C apparently reduced the viability of either the organisms or the seeds. Both plant fresh weight and root rot determinations showed a slight, but nonsignificant benefit from storing seeds at 5 than at 24 C (Fig. 9).

DISCUSSION

The success of the seed treatment method in biological control of seedling blight and root rot of pea relates to the technology of handling the organism before and after the seeds are coated. Of the factors that affect biological control, the amount of inoculum on the seed is important. Approximately  $6 \times 10^6$  spores per seed provided protection similar to that from captan, but the emergence

of seedlings when  $0.5 \times 10^6$  spores were applied per seed was not significantly different from that of nontreated seed. The number of spores per seed necessary to protect a plant can vary with the inoculum concentration of pathogens in the soil. Burr and Schroth (2) found that as concentrations of *Erwinia carotovora* increased, higher populations of the antagonist, a *Pseudomonas* sp., were needed to prevent rot on potatoes. When *E. carotovora* concentrations reached  $4 \times 10^5$  cells per potato disk, soft rot development was not eliminated by any concentration of the antagonist tested.

To determine the number of spores effective in biological control, it is necessary to know the number of viable spores and their germinability in soil and how many of these spores are needed to be effective at the seed surface. Fewer spores of *P. oxalicum* that were 125 days old were viable (70%) than spores that were younger, yet the older spores protected peas as well as did younger spores or captan. Also, there were  $3-4 \times 10^6$  fewer spores of *P. oxalicum* on seeds when the 125-day-old culture was used. To ensure protection, the seed may have to be "over-

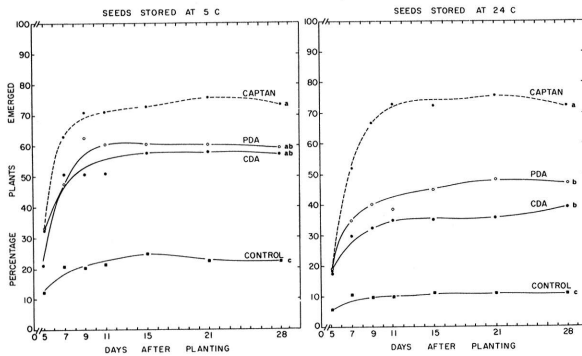


Fig. 8. Percentage emergence of plants from pea seeds stored at 5 and 24 C for 2 mo after seeds were coated with captan or with spores of *Penicillium oxalicum* grown on potato dextrose agar or Czapek-Dox agar and then planted in soil from the pea disease nursery in a greenhouse at  $21 \pm 4$  C. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .

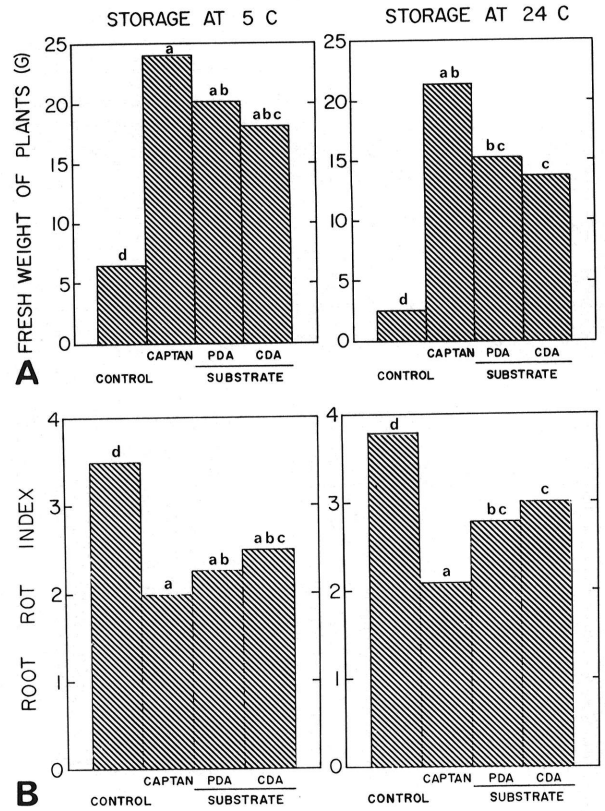


Fig. 9-(A, B). Effects of culture medium on inoculum production of *Penicillium oxalicum* and subsequent storage temperatures of treated seed as preplant treatments of pea seed. A) Fresh weight and B) root rot index of 25-day-old plants grown from pea seeds stored at 5 and 24 C for 2 mo after seeds were coated with captan or with spores of *Penicillium oxalicum* grown on potato-dextrose agar or Czapek-Dox agar and then planted in soil from the pea disease nursery in a greenhouse at  $21 \pm 4$  C. Means followed by the same letter are not significantly different according to Duncan's multiple range test,  $P = 0.01$ .

loaded" with spores because an insufficient number may germinate. Spores that are viable and germinable may not survive in soil either because of incompatibility with the host or environment, or, because of competition with other microorganisms in soil, including the pathogens; moreover the spores of the antagonist may be or become dormant.

*Penicillium oxalicum* was effective in preventing pre-emergence damping-off, but was less effective against postemergence damping-off which began about 3 wk after planting. Similarly table beets coated with spores of *Trichoderma viride* or *Penicillium frequentans* protected seedlings from preemergence damping-off (4). Protection has also been shown to be effective for 3-4 wk in corn (1) and for 2-3 wk in tomato (5).

Thus, the efficacy of *P. oxalicum* as an antagonist depends on numbers of spores per seed, storage temperature of treated seeds, and possibly substrate, but not on spore age. Even if a given factor has little influence, the cumulative effect is appreciable and this shows the importance of developing a technology to achieve the maximum biological control potential of microorganisms.

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