Effects of Pregermination of Pea and Cucumber Seeds and of Seed Treatment with *Enterobacter cloacae* on Rots Caused by *Pythium* spp.

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Research was supported in part by U.S.-Israel Binational Agricultural Research and Development (BARD) Fund Grant US-318-80.
Approved by the Director of the NY State Agricultural Experiment Station as Journal Series Paper 3497.
Pea seeds were provided by the Asgrow Seed Co., Kalamazoo, MI 49001 and cucumber seeds were supplied by Peto Seed Inc., Saticoy, CA 95695. Table beet seeds were provided by the Northrup King & Co., Gilroy, CA 95020.
Accepted for publication 13 April 1983.

ABSTRACT

Hadar, Y., Harman, G. E., Taylor, A. G., and Norton, J. M. 1983. Effects of pregermination of pea and cucumber seeds and of seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. Phytopathology 73:1322-1325.

In this work, we assessed the influence of germination prior to planting on susceptibility of seeds and seedlings to blights caused by soilborne *Pythium* spp., and determined biological events associated with changes in susceptibility. Cucumber and pea seeds were pregerminated in aerated water until radicle emergence. When pregerminated seeds were planted in soils infested with *Pythium* spp., disease incidence was greatly reduced relative to nonpregerminated seeds. When pregerminated seeds were planted in the presence of dead seeds or nongerminated pea seeds, more seeds rotted than when pregerminated seeds were planted in their absence. During seed germination in aerated water, 10^7–10^8 bacterial colony-forming units (CFU) were detected per milliliter of water, while approximately 10^7 CFU were detected per seed. Seeds germinated similarly, but under aseptic conditions, contained 20 or fewer bacteria per seed, while the water in which they were germinated contained fewer than 35 CFU/ml. Aseptically germinated seeds were more susceptible to *Pythium* spp. than seeds germinated under nonaseptic conditions. Dry seeds of cucumber, peas, and beets treated with the total bacterial population from germinated seeds were protected from rot. When one of these bacteria, *Enterobacter cloacae*, was used to treat pea, beet, or cucumber seeds, rots caused by *Pythium* spp. were markedly reduced. *E. cloacae* was the predominant bacterial species isolated from treated seeds 48 hr after planting in field soil. In vitro, *E. cloacae* formed sheaths of bacterial cells around hyphae of *P. ultimum*, and lysis of the enclosed hyphae resulted. There was no evidence of production of any diffusable antibiotic to *P. ultimum* by *E. cloacae*.

Additional key words: antagonism, *Beta vulgaris, Cucumis sativus,* fluid drilling, *Pisum sativum.*

Fluid drilling of germinated seeds is a method developed to improve seedling establishment and to increase the rate and uniformity of crop emergence (2). Seeds are germinated in aerated water until radicle emergence (pregerminated) and then transferred to a gel solution which prevents damage to the partially germinated seeds and which facilitates planting (17). The advantage of this system is that seeds are germinated in ideal conditions before sowing, thus eliminating the variable effect of the uncontrolled seedbed environment on germination (2).

There is little information on the influence of pregermination on the susceptibility of seedlings to seed rot. However, Short and Lacy (13) and Stasz et al (16) showed that pregerminated pea seeds are less susceptible to seed rot than are nongerminated seeds.

The purpose of this work was to assess susceptibility of pregerminated seeds to seedling blights caused by soilborne *Pythium* spp., and to determine the biological events associated with differences in susceptibility.

MATERIALS AND METHODS

Soil and pathogen. All experiments were performed in Arkport fine sandy loam field soil. The soil was obtained near Phelps, NY, and was stored in covered containers prior to use. *Pythium* spp. were those indigenous to Phelps soil; levels of this pathogen (colony-forming units [CFU]) were determined by dilution plating on the medium described by Mircetich (8). High levels of this pathogen (400–800 CFU per gram of soil) were obtained by cropping these soils with mixtures of wheat, barley, and oats for 3 wk, incorporating the crop into the soil, and incubating it for an additional 2 wk in the greenhouse. Greenhouse temperatures were maintained between 15 and 25 C. Soils with different levels of *Pythium* spp. were obtained by mixing cropped and noncropped soils.

Seeds and planting conditions. Nontreated seeds of peas (*Pisum sativum* L. 'Venus'), cucumber (*Cucumis sativus* L. 'Slice Master'), and table beets (*Beta vulgaris* L. 'Ruby Queen') were used in this study. Table beet seeds were sized to 4 mm prior to sowing. Five pea seeds or 10 beet or cucumber seeds were planted in the Arkport sandy loam per 10 × 10 × 6-cm plastic box, at a depth of approximately 1 cm. Consequently, pea seeds were spaced 3–5 cm apart, and beet or cucumber seeds were 1.5–2.5 cm apart. There were five boxes per treatment, and each box was considered to be a replication. The planted seeds were incubated in growth chambers at 25 C unless otherwise indicated. Gels used for planting of germinated seeds, at the rate of 1 ml per seed, were Laponite-508 (Laporte, Inc., Hackensack, NJ 07602) and Poly-Surf C (Hercules, Inc., Wilmington, DE 19899).

Seed germination. Seeds were germinated in glass columns (40.0 cm long and 5.2 cm in diameter) filled with 500 ml of distilled water. An airstone (aquarium aerator) at the bottom of each column provided aeration at approximately 500 ml/min. The flow rate was calculated from the time required to displace a known volume of water. The columns were maintained at 25 C in a constant-temperature water bath. Hereafter, seeds treated in such an apparatus are designated "pregerminated." Unless otherwise indicated, seed remained in the columns for 18–24 hr. Radicles emerged at 18 and 24 hr for cucumbers and peas, respectively. When aseptic germination was required, the columns were soaked with 1% NaOCl (prepared from a 5.25% solution of NaOCl, ie, laundry bleach) for 6 hr and then washed three times with sterile water. Seeds were surface sterilized in 1.75% NaOCl for 5 min and then rinsed with sterile distilled water prior to immersion in sterile distilled water contained in the sterilized columns. The air was filtered through a 0.45-μm (pore size) Millipore filter (Millipore Corp., Bedford, MA 01730) and the top of the column was plugged

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with cotton to prevent entry of airborne contaminants. This treatment is hereafter described as "aseptic." Seeds so treated were infested with very low levels (<10–20 CFU per seed) of microorganisms.

Selection of bacterial strains. Five grams of cucumber seeds were added to 500 ml of distilled water and mechanically shaken for 24 hr at room temperature (20–25 C), and then removed and replaced with 5 g of dry seeds. Shaking was resumed for an additional 24 hr. Samples of the water were diluted onto tripticase soy agar (Baltimore Biological Laboratories, Cockeysville, MD 21030) (TSA) plates so that <100 CFU per plate resulted after 48 hr of incubation at 25 C. The remainder of the water was centrifuged (5,900 *g* for 20 min) and the pellet containing the microorganisms was suspended in 4 ml of water. This suspension was used to coat dry cucumber seeds.

Hypahl mats of *Pythium ultimum* (isolate P4 of Pieczarka and Abawi [9]) were collected from malt extract broth cultures 48 hr after inoculation and were washed with sterile water. The collected hyphae were homogenized with sterile water in a Waring blender three times for 1 min each. This suspension was sprayed lightly over the surface of TSA dilution plates 48 hr after inoculation with dilutions of the water from germinating cucumber seeds. The plates were incubated at 25 C and observed daily under the microscope.

Identification of bacteria. To identify bacteria, we used the following tests: the Gram stain (15), the oxidase reaction (6), facultative anaerobic growth in NIH thioglycollate agar (Difco Laboratories, Detroit, MI 48233), the catalase reaction, the methyl red test (15), and the tests in the Oxiferm and Enterotube II tubes (Roche Diagnostics, Nutley, NJ 07110). The ability of bacteria to grow in the presence of KCN (15) and to hydrolyze chitin, tributyrin, gelatin, and Tween-80 also was determined (3). Flagellar arrangement was visualized by using a staining procedure (15). Identification was made according to the Roche Diagnostics Computer Coding and Identification System and was verified according to Sakazaki (10).

RESULTS

Effects of germination prior to planting on seed rots. When cucumber seeds were pregerminated for 24 hr and then planted in soil naturally infested with *Pythium* spp. (10 propagules per gram), no disease occurred (Fig. 1). More than 80% of nontreated seeds rotted under the same planting conditions. Significant disease reduction was observed when seeds were planted after only 2 hr of incubation in aerated water, and no disease occurred after 8 hr of incubation (Fig. 1). Similar results were obtained when germinated seeds were sown in gels used for fluid drilling. No increase in pre- or postemergence damping-off occurred when the propagule density was increased from 10 to 200 or 500 propagules of *Pythium* per gram of soil (*unpublished*). A decrease in soil temperature resulted in delayed emergence with both dry and germinated seeds (Fig. 2). However, pregermination greatly reduced disease incidence at both temperatures (Fig. 2).

When pregerminated peas were planted in soil infested with *Pythium* spp., disease incidence was reduced, but not eliminated (Table 1). As soil temperatures decreased, emergence was delayed. When air temperatures were cycled between 10 and 25 C (12 hr at each temperature), final stands from nontreated seeds were 40–50% lower than stands from pregerminated seeds (Table 1).

Cucumber and pea seeds were germinated on paper towels for 22 hr, killed by freezing at −20 C for 16 hr, and planted near dry or pregerminated seeds. The presence of dead seeds increased seedling

TABLE 1. Percentage of healthy pea seedlings and the time required to obtain 90% of maximum emergence for nontreated seeds or pregerminated pea seeds in soil infested with *Pythium* spp. and held at different temperature[a,b]

Parameters	Soil temperature (C)	Treated seeds[b]	Pregerminated seeds[b]
Healthy plants (%)	25	36	76
	20	40	84
	15	36	72
	10	36	76
Days for 90% of maximum emergence	25	4.5	2.6
	20	6.6	5.4
	15	7.0	5.6
	10	9.6	7.6

[a] Seeds were placed in aerated water maintained at 25 C until radicle emergence and then planted in Arkport sandy loam soil. All seed samples were capable of approximately 95% emergence from soil in the absence of *Pythium* spp.

[b] All comparisons between pregerminated and nontreated seeds at each temperature are significantly different according to Waller and Duncan's test ($K = 100$).

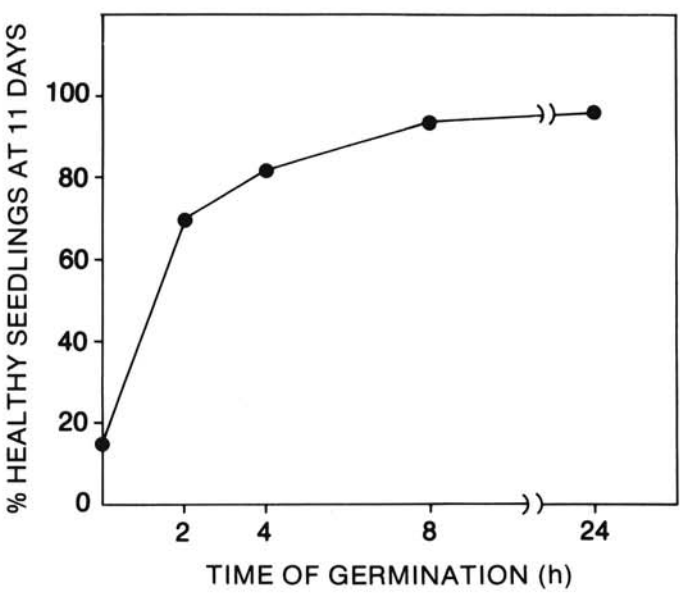

Fig. 1. The percentage of healthy cucumber seedlings produced from seeds pregerminated for various lengths of time and then planted in soil infested with *Pythium* spp. In the absence of *Pythium* spp. all seed samples were capable of emergence similar to that obtained by seeds pregerminated for 24 hr.

Fig. 2. Percentage of healthy cucumber seedlings produced from seeds germinated prior to planting in soil infested with *Pythium* spp., maintained at 25 C (▲) or in an incubator fluctuating between 10 and 20 C on a 12-hr cycle (●), and percentage of healthy seedling produced from non-germinated seeds at 25 C (△) or in a 10–20 C diurnal cycle (○). In the absence of *Pythium* spp., all seed samples were capable of approximately 95% emergence from soil.

disease both in cucumbers and peas (Table 2). Similar increases in cucumber postemergence damping-off were observed when dry pea seeds were planted together with pregerminated cucumber seeds.

The role of bacteria in the prevention of seed rot. Pea and cucumber seeds pregerminated aseptically contained only 10–35 bacteria per seed in comparison to 10^7–10^8 bacteria per seed for those pregerminated nonaseptically (Table 3). When pea seeds pregerminated aseptically were planted in soil infested with *Pythium* spp., levels of seed rot were similar to nongerminated seed controls. Cucumber seeds that had been aseptically pregerminated prior to planting developed levels of disease intermediate between those of dry seeds and of seeds nonaseptically germinated (Table 3).

Ninety-two percent of the cucumber seeds treated with bacterial suspensions (10^9/ml) from water used for pregermination of cucumbers produced healthy seedlings, but only 14% of the seedlings from nontreated seeds were healthy.

Isolation and identification of *Enterobacter cloacae*. After 3–5 days of incubation, the bacterial dilution plates that had been sprayed with hyphae of *P. ultimum* contained bacterial colonies that grew preferentially along the hyphal strands of *P. ultimum* (Fig. 3). No zones of inhibition were observed. Representative colonies were transferred to TSA and maintained for further study. Most of these bacterial colonies were composed of short rods and interacted similarly with *P. ultimum* (Fig. 3).

All of the bacterial colonies that grew preferentially along hyphal strands of *P. ultimum* consisted of short, motile, Gram-negative rods with peritrichous flagellae. Three typical isolates were all oxidase-negative, catalase-positive, facultative anaerobes. Gas and acid were produced from glucose, while acid was produced from arabinose, xylose, and sorbitol, but not from dulcitol after 24 hr of incubation. Acid was produced from lactose after 48 hr of incubation, but not after 24 hr. Isolates did not produce H_2S or indole from tryptophan, or pyruvic acid from phenylalanine. They did not produce lysine decarboxylase or urease, but did produce arginine dihydrolase and ornithine decarboxylase. Results of the Voges-Proskauer test were positive and those of the methyl red test were negative. Citrate was utilized as a sole carbon source and they grew on KCN. Tributyrin, Tween-80, and chitin were not hydrolyzed. There was slight gelatin liquification after 14 days of incubation. All these characteristics agree with the identification of the bacteria as *Enterobacter cloacae* (Jordan) Hormalche and Edwards (10). These strains of *E. cloacae* have been deposited in the USDA collection at the Northern Regional Research Center, Peoria, IL 61604, and are designated NRRL B-14095, NRRL B-14096, and NRRL B-14097.

***Enterobacter cloacae* as a biocontrol agent.** *E. cloacae* strain NRRL B-14095 was grown on King's B (5) broth for 18 hr. Cultures were twice centrifuged and washed with sterile water and suspended in sterile water. Beet, pea, and cucumber seeds were treated with the bacterial suspension and planted in soil infested with *Pythium* spp. The treatment resulted in greater than twofold increases in stands relative to nontreated seeds (Table 4). Bacterial populations, as determined by dilution platings on King's B medium, were 9×10^6 CFU per seed at the time of planting in Arkport sandy loam. After 24 hr in this soil, there were 10^8 CFU per seed, and after 48 hr there were 10^7 CFU per seed. Counts on tripticase soy agar were similar. Ten isolates from the 48-hr plates were tested using the Gram stain, the oxidase reaction, and the tests in the Enterotube II system. All of the isolates were similar in every respect to *E. cloacae*.

DISCUSSION

Pregermination of pea or cucumber seeds prior to planting in soil significantly reduced seed rots induced by *Pythium* spp. Planting of killed seeds produced a substrate for soilborne *Pythium* spp., and resulted in an increase in inoculum potential. When these killed

TABLE 2. Percentage of healthy seedlings produced from nongerminated or pregerminated and planted next to seeds killed[a] prior to planting in *Pythium*-infested soil

Treatments	Healthy seedlings (%)	
	Peas	Cucumber
Germinated seeds	76 a[b]	100 a
Germinated seeds + dead seeds	40 b	68 b
Nongerminated seeds	36 b	38 c
Nongerminated seeds + dead seeds	24 b	20 d

[a] Seeds were killed by freezing at −20 C for 16 hr after imbibition for 22 hr. Killed pea seeds were planted near normal pea seeds, and killed cucumber seeds were next to normal cucumber seeds.

[b] Means followed by the same letter within a column do not differ significantly according to Waller and Duncan's multiple range test (K = 100).

TABLE 3. The effect of pregermination conditions on amount of seed rot in soil infested with *Pythium* spp. and on numbers of bacteria on seeds and in water used to pregerminate seeds prior to planting

	Healthy seedlings[a]	Bacteria (CFU/seed)	Bacteria (CFU/ml)
Peas:			
Dry seeds	24 b	3×10^2	-
Germinated in non-aseptic conditions	68 a	1.2×10^7	1.3×10^8
Germinated in aseptic conditions	36 b	20	35
Cucumbers:			
Dry seeds	17 c	1.1×10^2	-
Germinated in non-aseptic conditions	100 a	1.7×10^7	2×10^7
Germinated in aseptic conditions	76 b	<10	<10

[a] Means followed by the same letter within a crop do not differ significantly according to Waller and Duncan's multiple range test (K = 100).

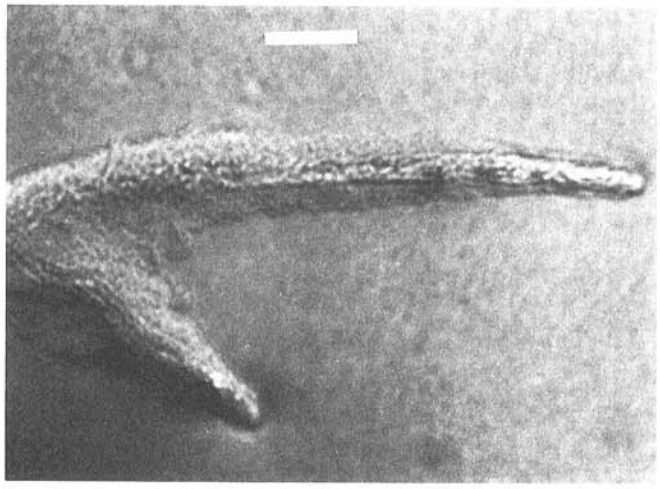

Fig. 3. Branched hyphae of *Pythium ultimum* sheathed in cells of *Enterobacter cloacae*. Both organisms were inoculated onto tripticase soy agar; this reaction was observed whenever the organisms were paired in culture.

TABLE 4. Protection of planted seeds of beets, peas, and cucumbers from rot by seed treatment with *Enterobacter cloacae*

Seed	Healthy seedlings produced from seeds (%)[a]	
	Nontreated control	Treated with *E. cloacae*
Beets	35 B	75 A
Peas	37 B	87 A
Cucumbers	40 B	92 A

[a] Means followed by the same letter within rows do not differ significantly according to Waller and Duncan's multiple range test (K = 100).

seeds were planted with pregerminated cucumber or pea seeds, damping-off of seedlings occurred with both crops, indicating that the seedlings from pregerminated seeds are not immune to the pathogen. These results are consistent with those of Short and co-workers (13,14) who showed that soaking pea seeds in water prior to planting reduced incidence of seed and seedling rot. They suggested that decreases in disease incidence occurred because of the removal of seed exudates which are stimulatory to pathogens (12). This suggestion was consistent with other observations that showed a correlation between the quantity of seed exudates and the level of seed rot (1,4,7,11).

However, in this work, both physiological changes in susceptibility and protection of seeds by bacteria were important in reducing seed and seedling rot. During the germination of both cucumber and pea seeds in aerated water columns, large numbers of bacteria grew in the water and on the seed surface. Seed exudates apparently formed a rich substrate for bacterial growth. Seeds pregerminated aseptically were more susceptible to *Pythium* than those pregerminated nonaseptically, but were more resistant than seeds not pregerminated prior to planting. When bacteria from germinated seeds were concentrated and coated on dry seeds, the seeds were protected from rot. Thus, the bacteria played a major role in the prevention of seed rot by germination of seeds prior to planting.

One of the more abundant bacteria in populations around germinating seeds was *E. cloacae*. It was able to protect seeds of cucumber, pea, and beet from *Pythium* seed rot, and is promising as a biocontrol agent for protection of seedlings. It persisted on seeds for at least 48 hr after planting, and other bacteria were rarely found on treated seeds. After this time, pea seeds are much less susceptible to *Pythium* spp. (16). The demonstration of the association of *E. cloacae* with protected seeds, its isolation in pure culture, the ability of pure cultures to protect seeds, and its reisolation from protected seeds (fulfilling Koch's postulates) demonstrates that *E. cloacae* is at least partially responsible for the decreased susceptibility of pregerminated seeds to seed rots. In culture, it forms sheaths around *P. ultimum* hyphae and causes lysis, but no antibiotic activity was evident. However, the mechanism by which *E. cloacae* protects seeds is not known. We frequently have noted sheathing by bacteria when attempting to isolate *Pythium* spp. from soil, suggesting that similar interactions may be a common phenomenon.

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