Biological Control of Rhizopus Rot of Peach with *Enterobacter cloacae*

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ABSTRACT

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Enterobacter cloacae (isolate D-3) delayed the onset and reduced the development of rot in artificially wounded peaches inoculated with *Rhizopus stolonifer*. *Rhizopus* infection was completely inhibited in 70% of fruit up to 5 days after inoculation. The effectiveness of *E. cloacae* was related to relative inocula concentrations of the pathogen and antagonist. Fruit firmness affected *Rhizopus* infection, but not *E. cloacae* effectiveness in controlling Rhizopus rot. Washed cells of *E. cloacae* were more effective than cells applied with the culture medium in which they grew. No compounds toxic to *Rhizopus* were detected in culture filtrates of *E. cloacae*.

Rhizopus spp. are major causes of postharvest fruit spoilage (1,5,7). On peach fruit (*Prunus persica* (L.) Batsch), it is second only to brown rot (*Monilinia fructicola* (Wint.) Honey) in causing postharvest losses. *Rhizopus* spp. are very aggressive wound parasites, which are difficult to control once fruit ripens.

Control of Rhizopus rot of peach fruits is presently accomplished with fungicides, hydrocooling, hot water dips, fungicide impregnated waxes, high temperature storage, and refrigeration (1,2,5,7,10,11). *Rhizopus stolonifer* (Ehr. & Fr.) Vuill. will not grow nor will spores germinate at temperatures below 7 C (5,11). Sensitivity of the organism to low temperatures has made precooling and refrigeration in storage and transit the primary methods of control. Unfortunately, when fruit is warmed above 7 C for marketing, the fungus can destroy it within 48 hr.

Dichloran (2,6-dichlora-4-nitroaniline) has been the fungicide of choice for *Rhizopus* control. Because most infections take place in wounds after harvest, postharvest treatments have been most effective. Dichloran is applied to peaches in waxes used on the processing line (7). Resistance to dichloran by *R. stolonifer* has been found (12). This fact plus pressure to reduce pesticides in the food chain increases the need for new postharvest disease control procedures for Rhizopus rot of peach.

Biological control of postharvest fruit diseases has recently met with considerable success (8,9,13). Pusey and Wilson (8) were able to control brown rot of peach with a strain of *Bacillus subtilis* (Ehrenberg) Cohn applied in waxes that are normally used on peaches when packed for fresh market distribution. *B. subtilis* controlled brown rot as well as the fungicide benomyl. Janisiewicz (6) has recently been able to control Botrytis and Penicillium rots of apple with antagonistic bacteria and yeasts.

Although we can biologically control brown rot of peach (8,9), fungicides still have to be applied to control Rhizopus rot when peaches are processed. This research was undertaken to develop a biological control procedure for Rhizopus rot of peach.

MATERIALS AND METHODS

More than 70 bacterial isolates were obtained on NYDA medium (8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose, and 15 g of bacto agar per liter, Difco, Detroit, MI) from soil and organic debris at the base of peach trees in the environs of Kearneysville, WV. These were screened in petri dishes for antagonistic activity against a *Rhizopus* sp. The *Rhizopus* isolate was obtained from rotting peaches. It was identified by the American Type Culture Collection as *R. stolonifer*. Zygospores were obtained when the isolate was mated with ATCC 622a (−strain) on PDA at 24 C. Bacterial isolates were streaked on one side of petri plates containing NYDA. An agar plug of *R. stolonifer* mycelium was placed on the other side. Thirteen isolates of bacteria that either inhibited growth and/or sporulation of *R. stolonifer* were selected for additional study. All isolates were stored on silica gel at −10 C.

Firm, ripe peach fruit were washed and surface sterilized in 10% sodium hypochlorite for the biocontrol tests. Wounds were made in the peaches 3 mm deep and 3 mm in diameter with a dissecting knife forced through a cork stopper. Potential bacterial antagonists were grown in 250-ml flasks containing 50 ml of NYDB (8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose,

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Difco, Detroit, MI) for 24 hr. One-milliliter aliquots of this culture were transferred to 200 ml of NYDB in 1-L flasks for 72 hr at 30 C. Subsequently, bacteria were centrifuged at 5,860 *g* for 20 min and resuspended in a volume of distilled water equal to the volume of culture filtrate before dilution. Bacterial suspensions used for the treatments contained approximately 1.5×10^{12} colony-forming units (cfu) per milliliter and were used immediately after they were diluted. Three serial dilutions were made of this concentration in distilled water to study the efficacy of *Enterobacter cloacae* Jorden, Hormaeche & Edwards. Culture filtrate was passed through a 0.20-μl filter (Nalge Company, Rochester, NY) before testing. Fruit that served as controls were also treated with distilled water and fresh NYDB.

Bacteria were applied to the entire fruit surface using a 3.8-cm nylon bristled paint brush. Ten fruit were used for each treatment. Special attention was made to cover the wound surface. *Rhizopus* spores were harvested from 3–7-day-old plate cultures using a Venturi spore trap or by agitating sporophores in sterile, deionized water and filtering the mycelium out with cheesecloth. *R. stolonifer* spore concentration was adjusted to 10^5, 10^4, or 10^3 spores per milliliter. Twenty microliters of aqueous spore suspension were pipetted directly into the wound approximately 2 hr after the bacterial inoculation. Dichloran was applied to the whole fruit at the rate of 0.12 g per 100 ml of water before inoculation with *R. stolonifer*. Test fruit were from the Appalachian Fruit Research Station or retail outlets. Peaches were randomly placed in plastic tubs with Styrofoam liners (Fig. 1B) and, after inoculation, stored at 22 C. Lesion size was measured along the fruit axis daily from 2 to 6 days.

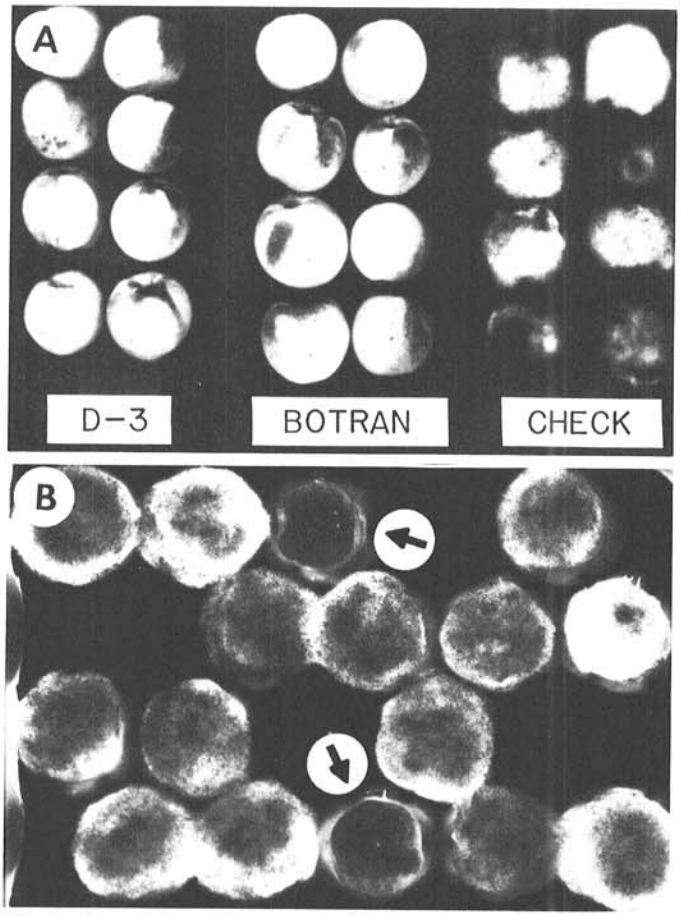

Fig. 1. Wounded peaches treated with bacterial cells, dichloran, and inoculated with *Rhizopus* sp. **A,** Wounded peaches treated with *Enterobacter cloacae* (D-3), dichloran, and a water check 5 days after inoculation with *Rhizopus* sp. **B,** Styrofoam tray holding one of 10 randomized treatments with 13 different bacterial isolates, dichloran, and a water check 5 days after inoculation. Only the peach at the top treated with *E. cloacae* (D-3) and the one at the bottom treated with dichloran were unrotted (arrows).

To determine the effect of firmness on infection, additional peaches were picked from a single tree on one day. They were divided into two firmness groups based on color and firmness (Table 1). Pressures were determined with a McCormick penetrometer model FT 327 fitted with a 0.79-cm tip. From each group, 20 fruit were wounded and treated with bacteria and 20 were left untreated. *Rhizopus* inoculum was at 10^5 spores per milliliter.

RESULTS

Among the 13 bacterial isolates tested on peach fruit against *R. stolonifer*, only one reduced rot development comparable to dichloran (Fig. 1; Table 2). This experiment was conducted three times with similar results. The single effective bacterium was identified as typical of *E. cloacae* by the American Type Culture Collection (SC 843). Cells are gram-negative rods and motile. Colonies are entire, glistening, smooth, and mucoid and the flagella peritrichous on PDA agar medium. The *B. subtilis* isolate used for the control of brown rot was not successful in controlling Rhizopus rot (Table 2).

Infections by *R. stolonifer* were inhibited by *E. cloacae* in 70% of the fruit and the onset of rot in succumbing fruit was slowed in treated versus untreated fruit (Fig. 1; Table 2). Decay was significantly reduced from 2 through 5 days following inoculation. Most fruit treated with *E. cloacae* eventually developed *Rhizopus* lesions after 8 days.

The filtrate from cultures of *E. cloacae* accelerated colonization by *R. stolonifer* compared with a distilled water control. Cells of *E. cloacae* resuspended in a comparable volume of deionized water were more effective in controlling *Rhizopus* than those applied in the culture medium. This indicates that *E. cloacae* does not produce an antibiotic, as is the case with *B. subtilis* against the brown rot pathogen (8).

TABLE 1. Mean diameter of rot on peaches treated with *Enterobacter cloacae* or water, and inoculated with *Rhizopus* spores[z]

Treatment	Mean pressure at inoculation (kg)	Fruit with lesions (%)	Mean diameter of lesion (mm)
Water check	2.0	80	88.3 a
Water check	4.5	45	53.8 b
E. cloacae	4.4	15	11.6 c
E. cloacae	1.7	5	1.7 c

[z]Means not followed by the same letter are significantly different ($P<0.05$) according to Duncan's multiple range test.

TABLE 2. Mean diameter of rot infected area after 4 days on peaches treated with different bacterial isolates and inoculated with *Rhizopus* spores[z]

Treatment	Isolate number	Mean diameter of lesion (mm)
Water check		200 a
Media check		200 a
Isolate	(D-11)	200 a
Isolate	(D-2)	200 a
Isolate	(D-8)	200 a
Isolate	(D-10)	200 a
Isolate	(D-12)	185 a
Isolate	(D-7)	185 a
Isolate	(D-4)	170 ab
Bacillus subtilis	(B-3)	157 abc
Isolate	(D-9)	154 abc
Isolate	(D-5)	152 abc
Isolate	(D-1)	126 bc
Isolate	(D-6)	114 c
Enterobacter cloacae	(D-3)	11 d
Dichloran 75WP (450 mg/L)		1 d

[z]Means not followed by the same letter are significantly different ($P<0.05$) according to Duncan's multiple range test.

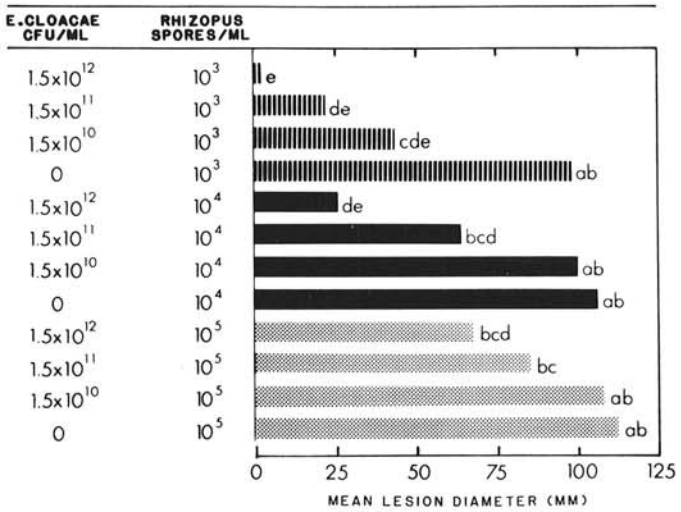

Fig. 2. Wounded peaches treated with different inocula concentrations of *Enterobacter cloacae* and *Rhizopus stolonifer*. Mean lesion diameters not followed by the same letter are significantly different ($P < 0.05$) according to Duncan's multiple range test.

Concentrations of both the pathogen and the antagonist affected Rhizopus rot development (Fig. 2). A 10- and 100-fold dilution of *E. cloacae* cells allowed more infection than undiluted cells (1.5×10^{12} cfu). Only at spore levels 10^3 of *R. stolonifer* spores per milliliter was the 100-fold dilution significantly different from a water control. The 10^{-1} dilution had noticeably less infection than the control at each inoculum level, but was only significantly different at 10^3 spores per milliliter. Undiluted cells gave significantly better control than a water check at both 10^3 and 10^4 spores per milliliter, but not at 10^5 spores per milliliter (Fig. 2). The ability of *E. cloacae* to prevent decay decreased with increasing amounts of *Rhizopus* inoculum. The percentage of peaches treated with undiluted *E. cloacae* developing lesions after 5 days was 10% with 10^3 spores per milliliter, 30% with 10^4 spores per milliliter, and 80% with 10^5 spores per milliliter. Unprotected peaches had 80% lesion development with 10^3 spores per milliliter, 100% with 10^4 spores per milliliter, and 90% with 10^5 spores per milliliter (Fig. 2).

Control was independent of fruit firmness as measured by fruit pressure. Less firm fruit (2-kg pressure) were more susceptible to *Rhizopus*. However, control with *E. cloacae* was equally effective at all pressures tested (Table 1).

DISCUSSION

Complete control of Rhizopus rot of peaches was not obtained with *E. cloacae*. However, the test conditions used are more favorable for Rhizopus rot than those found commercially. The effectiveness of *E. cloacae* can probably be enhanced through more refined preparation and application procedures of the bacterial inoculum.

E. cloacae is widely distributed in nature. It is found on grains and plants, in water, milk, dairy products, and the intestinal tract of man and other animals (3). The use of *E. cloacae* as a biocontrol agent on food will require careful attention to possible adverse effects on man and other animals. The fact that this organism is already a normal inhabitant of man's intestinal tract indicates that it may be safe.

Possibly *E. cloacae* could have a wide application in biological control. Hadar et al (4) recently demonstrated partial control of pea and cucumber seedling rots caused by *Pythium* spp. with *E. cloacae*. In this instance, the antagonist was not found to produce an antibiotic against *Pythium* spp. but formed a sheath around the hyphae and lysed them. Our preliminary observations with *E. cloacae* against *R. stolonifer* (*unpublished*) indicate a similar mechanism.

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