Bacterization of Potatoes with *Pseudomonas putida* and Its Influence on Postharvest Soft Rot Diseases

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ABSTRACT

Colyer, P. D., and Mount, M. S. 1984. Bacterization of potatoes with *Pseudomonas putida* and its influence on postharvest soft rot diseases. Plant Disease 68:703-706.

Preplant treatments of potato seed pieces (cultivar Superior) and postharvest treatments of potato tubers with a *Pseudomonas putida* isolate antagonistic to *Erwinia* spp. were evaluated for their effect on soft rot development. The percent weight loss due to soft rot, the surface area and volume of tubers with rot, and the number of toothpick wounds from which rot developed were reduced by 50% in preplant treatments and by 75% in postharvest treatments. Greater soft rot reduction in postharvest treatments may have been the result of greater colonization of the tubers by *P. putida*.

Pseudomonads are the largest group of microorganisms that produce antibiotics (14). Many of these compounds have inhibitory activity against plant pathogens (2,4,15,28). Some of the pseudomonads that produce antibiotics have been used in disease control. Damping-off of cotton (8,9) and onions (11) has been successfully controlled through the application of pseudomonads. Antagonistic pseudomonads also have reduced the incidence of bacterial blotch of mushrooms (19), bacterial blight of beans (24), and Dutch elm disease (22). Reduced populations of *Erwinia* spp. were recorded from potato tubers grown from seed tubers treated with antagonistic pseudomonads, but the effect on postharvest soft rot development was not investigated (12). The objective of this research was to investigate the ability of a *Pseudomonas putida* isolate with inhibitory activity against soft-rotting *Erwinia* spp. to reduce postharvest soft rot of potatoes.

MATERIALS AND METHODS

Bacterial strains. *P. putida* (M17), used throughout this study, was isolated from healthy tomato fruit on nutrient agar.

This strain was identified as a *P. putida* on the basis of fluorescent pigment production on King's agar B, positive oxidase reaction, negative gelatin hydrolysis, positive arginine dihydrolase activity, and multiple polar flagella.

An antibiotic negative mutant (M74) was produced by treatment of *P. putida* (M17) with nitrosoguanidine. The antibiotic negative mutant was used to determine if the effect of bacterization on soft rot development was associated with antibiotic production by the bacterium. The mutation procedure was a modification of the technique outlined by Adelburg et al (1). *P. putida* (M17) was grown for 18 hr in a glycerol-minimal salts medium. The minimal salts basal medium (0.15% KH_2PO_4, 0.715% Na_2HPO_4, 0.3% $(NH_4)_2SO_4$, 10% glycerol, and 1.0% $MgSO_4 \cdot 7H_2O$ were prepared and autoclaved separately. After autoclaving, glycerol and magnesium sulfate were added to the minimal salts medium at a final concentration of 1 and 0.02%, respectively. Colonies resulting from mutation were replica-plated and tested for antibiotic production using the overlay technique of Vidaver et al (27).

Cultures of M17 and M74 were genetically marked for resistance to the antibiotics rifamycin and nalidixic acid according to the procedure of Kloepper et al (13) to follow colonization of potato roots, stolons, and daughter tubers. Rifamycin- and nalidixic acid-resistant colonies of similar size and shape as the wild-type bacteria were selected and tested for production of antibiotic activity using the overlay technique with *Erwinia carotovora* (EC14) as the indicator.

Erwinia strains used for the growth inhibition studies are listed in Table 1. These strains were maintained on nutrient agar at 30 C. The pseudomonad cultures were maintained on *Pseudomonas* agar F (Difco) at 24–32 C.

Growth inhibition of *Erwinia* spp. The ability of *P. putida* (M17) to inhibit the growth of several plant-pathogenic *Erwinia* spp. was tested using the overlay procedure outlined by Vidaver et al (27). One drop (about 0.05 ml) of *P. putida* (M17) from an 18-hr nutrient broth culture was spotted in the center of a petri plate containing about 25 ml of nutrient agar (Difco). After incubation for 48 hr at 24 C, the plates were exposed to chloroform vapors. Screw-cap culture tubes containing 3.2 ml of sterile 0.5% water agar were inoculated with 0.1 ml of an 18-hr nutrient broth culture of the indicator bacterium, poured over the killed *P. putida* (M17) cultures, and incubated at 24 C. After 24 hr, the zone of inhibition was measured from the edge of the *P. putida* (M17) colony to where the indicator lawn began. All tests were done in triplicate. The indicator bacteria are listed in Table 1.

Table 1. Growth inhibition of selected *Erwinia* strains by *Pseudomonas putida* (strain M17)[a]

Organism	Source	Strain	Zone size (mm)
E. carotovora pv. *atroseptica*	A. Kelman	SR8	11.8
E. carotovora pv. *carotovora*	G. Lacy	E129	12.9
E. carotovora pv. *carotovora*	R. Dickey	EC14	15.4
E. carotovora pv. *carotovora*	R. Dickey	EC311	13.4
E. carotovora pv. *carotovora*	A. Kelman	SR17	11.5
E. carotovora pv. *carotovora*	G. Lacy	SR53	17.5
E. carotovora pv. *carotovora*	A. Kelman	SR259	14.0

[a] Determined by the overlay technique of Vidaver et al (28). Zones were measured from the edge of the *P. putida* (M17) spot to the lawn edge of the indicator bacterium.

Planting and bacterization of seed pieces. Potatoes were planted in a 10 × 6 randomized block design (three treatments × two replicates). Each block (three rows) measured about 2.8 m × 4.6 m. Spacing between individual plots was 1 m on every side. Three treatments were used to examine the effect of preplant seed treatments on the soft rot development of potato tubers at harvest: 1) control, 2) tubers treated with *P. putida* (M17), and 3) tubers treated with *P. putida* (M74). The antibiotic-negative mutant was included to determine if any differences between the control and the *P. putida* (M17) (antibiotic-positive) treatment could be attributed to the antibiotic-producing capability of the introduced bacterium.

Bacterial suspensions for bacterization were prepared in the following manner: Several liters of *P. putida* (M17 and M74) were grown for 48 hr at 28 C in nutrient broth amended with 0.4% technical casamino acids (Difco) on a rotary shaker. The bacteria were pelleted by centrifugation at 10,000 g and resuspended in distilled water. Resuspended bacteria were adjusted with distilled water to populations of 10^8 colony-forming units (cfu) per milliliter as determined spectrophotometrically at 560 nm. Seed tubers were cut and placed in the appropriate bacterial suspension for 15–30 min before planting.

In late August, the vines were killed, and the tubers were harvested manually and stored at 12–16 C in 95–98% relative humidity to encourage suberization (17). About 2 wk later, the temperature was lowered to 2–5 C to promote longer storage life of the tubers.

Colonization of potato rhizoplanes by *P. putida* (M17). Colonization of potato roots by *P. putida* (M17) was determined with the antibiotic-resistant mutants. Beginning 2 wk after plant emergence, one plant from each treatment was unearthed and placed in a 6-in. pot. The pots were taken to the laboratory and excess soil was removed from the roots and daughter tubers. Ten grams of root tissue was placed in a flask containing 100 ml of sterile distilled water and incubated 1 hr.

After incubation, the flasks were

placed on a Vortex for 1 min, and the resulting suspension was serially diluted. One-tenth of a milliliter of each dilution was spread on each of three *Pseudomonas* agar F plates amended with 100 μg/ml of rifamycin and 100 μg/ml of nalidixic acid. After 48 hr, colonies characteristic of *P. putida* were counted. This procedure was repeated weekly for 10 wk.

Populations of *P. putida* (M17) on daughter tubers were also determined periodically during storage. The same procedure used for determining populations of *P. putida* (M17) on the roots was employed except 10 g of potato peel was placed in the sterile distilled water.

Soft rot evaluation. The influence of bacterization by *P. putida* (M17) on the postharvest development of soft rot was evaluated according to a modification of the procedures of DeBoer and Kelman (6) and Perombelon (21).

Potato tubers from each preplant treatment were removed from storage, weighed, wounded 10 times each with sterile wooden toothpicks at a depth of about 5 mm, and covered with a film of water. The tubers were then incubated at 22 C in an anaerobic chamber (Gas Pak Anaerobic Systems, BBL Industries). After 5 days, tubers were removed and washed under a steady stream of water to remove any rotted tissue.

Severity of soft rot was evaluated by a modification of the techniques used by Lund and Kelman (16). Three parameters were employed for evaluation of soft rot severity: 1) percent weight loss determined by weight change of the tuber before and after anaerobic incubation divided by weight before incubation, 2) visual assessment of the surface area and volume of soft rot based on a rating scheme where 0 = no rot and 5 = complete rot, and 3) the number of toothpick wounds from which soft rot developed.

In the evaluation of postharvest treatments of tubers with *P. putida* (M17) for soft rot content, tubers were taken from storage and placed for 10–15 min in 48-hr suspensions of *P. putida* (M17) in nutrient broth amended with 0.4% casamino acids. Tubers placed in distilled water served as controls. Soft rot severity was assessed as described in the preplant treatments.

Effect of incubation of tubers inoculated with *P. putida* (M17) after harvest on soft rot potential. The influence of postharvest inoculation of tubers with *P. putida* (M17) on soft rot potential over time was evaluated. Tubers were placed for 10–15 min in bacterial suspensions of *P. putida* (M17) either in distilled water or in nutrient broth and stored without drying at 5 C and 95% relative humidity. Tubers placed in distilled water served as controls. After 1 and 5 days, the tubers were evaluated for soft rot severity as described before.

The number of *P. putida* that colonized the peel after 1 and 5 days of storage was also determined. Ten grams of potato

peel was placed in 100 ml of sterile distilled water. After 1 hr of incubation, the flasks were placed on a Vortex, the resulting suspension diluted, and 0.1 ml of each dilution spread on each of three *Pseudomonas* agar F plates.

Sensitivity of pectolytic bacteria to *P. putida* (M17). Soft rot lesions were observed on tubers treated with *P. putida* (M17). To determine whether these rots developed because of insufficient protection by *P. putida* (M17) or because of soft-rot bacteria resistant to the antibiotic produced by M17, bacteria from rotted areas were evaluated for sensitivity to M17 using the overlay technique.

Loopfuls of tissue from rotted areas of *P. putida* (M17)-treated tubers were suspended in 10 ml of sterile distilled water. The suspension was serially diluted two or three times and 0.1 ml of each dilution spread on each of three crystal-violet polypectate agar plates (5). Bacteria that caused depressions on the agar were streaked on nutrient agar. Isolated colonies were tested for pectolytic activity on crystal-violet polypectate agar and for susceptibility to the antibiotic compound produced by *P. putida* (M17).

RESULTS

Suitability of *P. putida* (M17) as a bacterization agent. *P. putida* (M17) showed strong and uniform inhibitory activity against the growth of various *Erwinia* strains. The size of the zones of inhibition are presented in Table 1. The degree of antibiotic sensitivity for the bacteria was similar.

Bacterization studies. *P. putida* (M17) colonization of the rhizoplane. *P. putida* (M17 and M74) were able to colonize the rhizoplane of field-grown potato plants (Fig. 1). Colonization of potato rhizoplanes for M17 and M74 was similar. From shoot emergence to 1 mo later, population levels were about 10^5 cfu/g of root. When measurements were taken on 14 July, population densities had declined to 10^2 cfu/g of root and remained at this level for the remainder of the experiment (29 July). Populations of *P. putida* (M17) on daughter tubers were variable (between 0 and 10^3 cfu/g of peel) but averaged 50 cfu/g of peel.

Soft rot evaluation. In 1980 and 1981, the percent rotted tissue of daughter tubers from seed tubers treated with *P. putida* (M17) was significantly lower than for control tubers (Table 2, Fig. 2). Tubers grown from seed tubers treated with *P. putida* (M74) were intermediate in percentage of rotted tissue. Tubers produced by M17-treated seed tubers were significantly lower in visual rating and number of toothpick wounds from which rot developed than tubers produced by M74-treated and control seed tubers. In 1982, similar results were obtained in soft rot evaluation data; however, *P. putida* (M74) did not
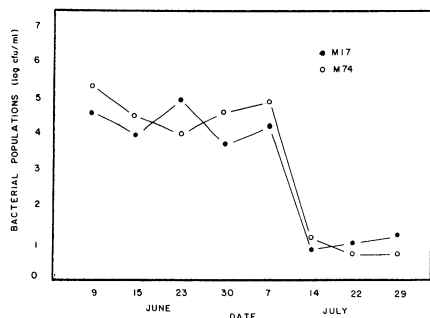

Fig. 1. Survival of *Pseudomonas putida* (isolates M17 and M74) in the rhizoplane of field-grown potato plants (cultivar Superior).

Table 2. Influence of preplant treatments with *Pseudomonas putida* on soft rot development of potato tubers (cultivar Superior)[w]

| | 1980 | | | 1981 | | | 1982 | | |
Treatment[x]	Rotted tissue[y] (%)	Visual rating[y]	No. lesions/ tuber[y]	Rotted tissue (%)	Visual rating	No. lesions/ tuber	Rotted tissue (%)	Visual rating	No. lesions/ tuber
M17 (anti +)	8.1 a[z]	1.5 a	4.1 a	8.1 a	1.9 a	5.3 a	6.7 a	1.8 a	5.3 a
M74 (anti −)	13.7 a	2.6 b	6.1 b	11.3 a	2.5 b	6.8 b	10.7 ab	2.7 b	7.0 b
Control	26.3 b	3.7 c	8.7 c	14.9 b	3.3 c	8.2 c	15.7 b	3.3 b	8.3 b

[w] 1980 Data based on 34 tubers per treatment, 1981 data based on 61 tubers per treatment, and 1982 data based on 20 tubers per treatment.

[x] M17 (anti +) = *P. putida* (M17) with inhibitory activity against *Erwinia* spp. M74 (anti −) = *P. putida* (M74) without inhibitory activity against *Erwinia* spp. Control = no treatment.

[y] Rotted tissue (%) = amount of rotted tissue determined by weight change before and after soft rot evaluation. Visual rating: 0 = no rot to 5 = complete rot, based on visual estimate of surface area and volume rotted. No. lesions = number of toothpick wounds from which soft rot developed, 10 possible per tuber.

[z] Means followed by the same letter are not significantly different (*P* = 0.01) according to Duncan's multiple range test.

produce values that were significantly different from control tubers.

Postharvest treatment of tubers with *P. putida* (M17) gave greater reductions in soft rot development than preplant treatments (Tables 2 and 3). Tubers from preplant treatments of tubers with *P. putida* (M17) averaged about 8.0% rotted tissue compared with 4.5% for postharvest treatments.

Similar results were obtained for postharvest treatments in 1981 and 1982 (Table 3). Significant differences in percent rotted tissue, visual rating, and number of toothpick wounds from which rot developed were observed between *P. putida* (M17)-treated and control tubers in both years.

Effect of storage of tubers inoculated with *P. putida* (M17) after harvest on soft rot development. Postharvest treatments were effective in reducing the severity of soft rot even though the tubers were stored an additional 1 and 5 days after inoculation with *P. putida* (M17) before soft rot evaluation (Table 4). The percentages of rotted tissue, visual ratings, and number of toothpick wounds from which rot developed for 1- and 5-day storages, according to regression analysis, were not significantly different from values obtained without the additional storage.

P. putida (M17) was able to colonize tubers at populations of 4.0×10^5 cfu/g of peel after 1 day of storage and at 4.5×10^4 cfu/g of peel after 5 days of storage (Table 4). Although populations were slightly reduced after 5 days, this reduction did not result in an increase in severity of soft rot.

Sensitivity of pectolytic bacteria to *P. putida* (M17). Sixty-two pectolytic bacterial isolates obtained from rotted lesions of *P. putida* (M17)-treated tubers were evaluated for their sensitivity to the inhibitory compound produced by *P. putida* (M17). No attempt was made to determine whether these pectolytic isolates were in fact *Erwinia* spp. or to test their pathogenicity (soft rot ability) on potatoes. About 60% of these isolates were inhibited by *P. putida* (M17). The inability of *P. putida* (M17) to provide complete control of soft rot may be
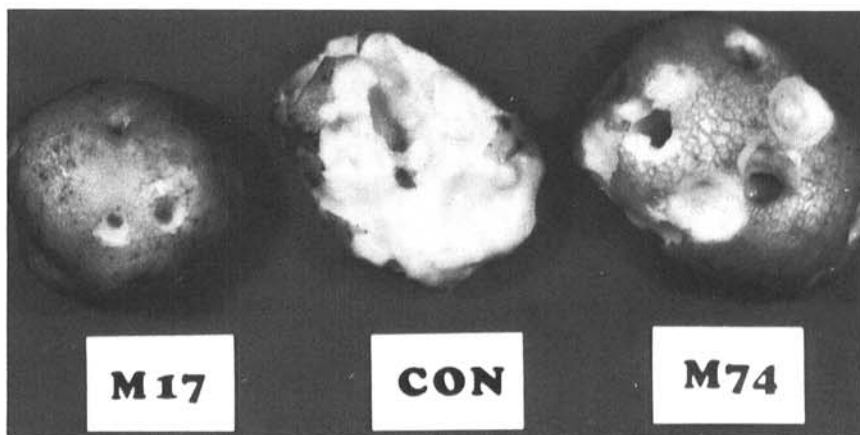
Fig. 2. Appearance of potato tubers after soft rot evaluation. Tubers treated with *Pseudomonas putida* (M17) (antibiotic-positive), *P. putida* (M74) (antibiotic-negative), and distilled water (control).

Table 3. Influence of postharvest treatments with *Pseudomonas putida* (M17) on soft rot of potato tubers (cultivar Superior)[w]

| | 1981 | | | 1982 | | |
Treatment[x]	Rotted tissue[y] (%)	Visual rating[y]	No. lesions/ tuber[y]	Rotted tissue (%)	Visual rating	No. lesions/ tuber
M17	3.3 a[z]	1.2 a	2.2 a	5.3 a	1.7 a	5.0 a
Control	11.9 b	2.9 b	8.0 b	12.5 b	3.2 b	8.1 b

[w] 1981 Data based on 50 tubers per treatment, 1982 data based on 17 tubers per treatment.

[x] M17 = *P. putida* (M17) with inhibitory activity against *Erwinia* spp.

[y] Rotted tissue (%) = amount of rotted tissue determined by weight change before and after soft rot evaluation. Visual rating: 0 = no rot to 5 = complete rot, based on visual estimate of surface area and volume rotted. No. lesions = number of toothpick wounds from which soft rot developed, 10 possible per tuber.

[z] Means followed by the same letter are not significantly different (*P* = 0.01) according to Duncan's multiple range test.

related to the presence of antibiotic-resistant pectolytic bacteria.

DISCUSSION

Several researchers have successfully employed bacterization with pseudomonads to control plant diseases (8,9,11,18,19). The ability of *P. putida* (M17) to inhibit *E. carotovora* strains and its nonpathogenicity on potato make it a good agent to study bacterization of potato. The inability to verify the successful establishment of introduced bacteria in the rhizoplane has been cited as a limitation of bacterization experimentation (10,13,23). The ability of *P.*

putida (M17) to become established in potato rhizoplanes was verified using antibiotic-resistant markers.

Daughter tubers derived from seed tubers treated with *P. putida* (M17) and tubers treated with *P. putida* after harvest showed less soft rot than control tubers. Tubers produced by seed tubers treated with the antibiotic-negative *P. putida* (M74) gave intermediate reduction in soft rot severity. Abiosis may be responsible for the inability of the antibiotic-negative bacterium (M74) to reduce soft rot severity to the same level as the antibiotic-positive bacterium (M17). However, proof of antibiotic production in the soil

Table 4. Effect of 1- and 5-day incubation periods of harvested potato tubers (cultivar Superior) inoculated with *Pseudomonas putida* (M17) before soft rot evaluation

Evaluation parameter[a]	Treatment[b]	Incubation (days)		
		0	1	5
Rotted tissue (%)	NB	3.4	3.6	3.3
	DW	6.3	3.5	4.2
	CON	10.9	11.8	12.1
No. lesions/tuber	NB	5.3	4.6	3.6
	DW	5.7	4.0	4.8
	CON	7.4	8.3	7.6
Visual rating	NB	1.7	1.4	1.4
	DW	1.9	1.7	1.9
	CON	3.1	3.1	3.0
		Population[c]		
Survival of *P. putida*	NB	8.0×10^8	4.9×10^5	4.7×10^4
	DW	8.0×10^8	3.5×10^5	4.2×10^4
	CON	...	...	...

[a] Rotted tissue (%) = amount of rotted tissue determined by weight change before and after soft rot evaluation. Visual rating: 0 = no rot to 5 = complete rot, based on visual estimate of surface area and volume rotted. No. lesions = number of toothpick wounds from which soft rot developed, 10 possible per tuber.
[b] Data based on seven tubers per treatment. NB = application of *P. putida* (M17) in nutrient broth, DW = application of *P. putida* (M17) in distilled water, CON = control.
[c] Survival of *P. putida* in colony-forming units per gram of peel. Values at 0 days are populations in initial inoculum.

has not been established. Production of antibiotics on culture media is not indicative of their production in the soil (3,20). In fact, some antibiotics are produced only in culture (25). Conclusions based on the reduction of disease and a corresponding increase in the population of the introduced antibiotic-producing bacterium, as observed in this research, are circumstantial (7,20). Furthermore, there may be a dual role of competition and abiosis in the reduction of soft rot severity. Another mechanism, not investigated, could be induced resistance. Proof of antibiotic production in the soil is needed before disease suppression can be attributed to abiosis.

In postharvest treatments, the soft rot development was reduced to a greater extent than in preplant treatments. This was probably the result of greater colonization of the tubers by *P. putida* (M17) and, as a result, better protection of the tubers. *P. putida* populations up to 10^4 cfu/g of peel colonized tubers up to 5 days after postharvest treatments, compared with populations of 50 cfu/g of peel on daughter tubers grown from seed pieces treated at planting.

The duration of effectiveness of postharvest treatments on soft rot development is very important for some commercial applications. Significant reductions in soft rot severity and colonization of tubers by *P. putida* (M17) were observed on tubers treated 5 days before soft rot evaluation (Table 4). Although 5 days represents only a small portion of the actual storage period for potatoes, it does demonstrate the ability of *P. putida* (M17) to survive under storage conditions for short periods.

The strictly controlled conditions under which fruits and vegetables are stored is one of the advantages of postharvest inoculation with biological control agents. For most crops, including potatoes, these controlled conditions include high relative humidities that would prevent desiccation of biological control agents. Although temperatures are often low enough to slow microbial growth, they are not fatal (26). Exploitation of these storage conditions to enhance the survival of biological control agents may provide more effective control.

The inability of *P. putida* (M17) to provide complete control of soft rot may be related to incomplete colonization by the bacterium (ie, areas colonized by soft-rotting *Erwinia* spp. that are not accessible to *P. putida*). Also, pectolytic bacteria resistant to the antibiotic produced by *P. putida* (M17) were found in rotted lesions of *P. putida*-treated tubers. The occurrence of these resistant populations is one of the limitations of *P. putida* (M17) as a biological control agent for soft rot diseases. The inability of *P. putida* (M17) to provide absolute control is probably a result of a combination of these factors.

In summary, treatment of potatoes with a *P. putida* isolate with antagonistic activity toward soft-rotting *Erwinia* spp. was successful in reducing the soft rot development in potato tubers.

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