

Antagonistic Effect of Soil Bacteria on *Fusarium roseum* 'Culmorum' from Carnation

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ABSTRACT

Bacteria, isolated from soil, were antagonistic to *Fusarium roseum* 'Culmorum' in vitro and in vivo. Unrooted carnation cuttings were inoculated with antagonistic bacteria and rooted in the presence of the pathogen. The bacteria reduced the number and development of basal stem lesions, but did not inhibit rooting during the propagation period. One bacterial isolate (isolate A) was more effective than were nine other isolates. Carnation cuttings

inoculated with isolate A were free of basal stem lesions 4 months after inoculation of the cuttings with *F. roseum* 'Culmorum', yet the pathogen was recovered from these cuttings. The general characteristics of the bacterium, designated isolate A, place it in the family Pseudomonadaceae.

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Fusarium roseum (Lk.) emend. Snyd. & Hans. 'Culmorum' (14) causes a basal stem rot of carnation (*Dianthus caryophyllus* L.) cuttings during propagation. Cultural practices and chemical treatments have been suggested as control measures (11). Antagonistic microorganisms also have been tested as a means of controlling *F. roseum* (1, 7) and other plant pathogens (18, 19). Wood & Tveit (20), Garrett (5), and Snyder (13) reported that microorganisms which adversely affect the growth of plant pathogens may function in plant disease control. Several workers used antagonistic microorganisms in control of *Fusarium* stem and root rots of cereals (4, 15, 17) and damping-off of seedlings (8, 21); seed-coating techniques were used. Since little work has been reported on the use of antagonistic microorganisms to reduce the stem rot disease of carnation cuttings caused by *F. roseum* 'Culmorum' (Fig. 1), we (i) examined soils for naturally occurring antagonistic microorganisms; (ii) tested these soil microorganisms for antagonism to *F. roseum* 'Culmorum' in vitro; (iii) tested the antagonistic microorganism's ability to reduce the development of stem lesions on unrooted carnation cuttings; and (iv) determined whether the antagonistic microorganisms remained active against the pathogen when introduced into carnation cuttings.

MATERIALS AND METHODS.—*Isolation of antagonistic bacteria from soil.*—Soil samples were collected from 39 field locations, stored in polyethylene bags at room temperature, and used to prepare soil dilution plates (6). Soil extract agar (SEA) (6, 9), SEA plus 0.1% Difco peptone and 0.1% dextrose (SEA + PD), potato-dextrose agar (PDA), and Difco nutrient agar (NA) were used for isolation and culture of soil microorganisms.

Soil dilution plates were prepared with SEA, NA, and SEA + PD using 1:10⁵ and 1:10⁶ dilutions of a

1-g sample of field soil in sterile distilled water, and were incubated at 23 ± 1 C for 24 hr. An aqueous suspension of macroconidia of *F. roseum* 'Culmorum' (R-72) then was atomized on the agar surface. Isolate R-72, utilized throughout this study, was obtained from a carnation plant, cultivar Laddie Sim, grown in Salinas, Calif. The suspension was prepared by using 100 ml of sterile water to wash the conidia from a culture initiated from a single conidium grown in PDA in a test tube under artificial light (16) at 23 ± 1 C for 18 to 21 days.

If zones of inhibition occurred within 14 days on soil dilution plates inoculated with R-72 (Fig. 2), bacteria from colonies within the zones were removed and streaked on NA plates, and single colonies were transferred to NA and SEA plates and slants. Zones were either large (i.e., 3 mm or greater in diameter and with a distinct margin and few hyphae entering the clear zone) or small (i.e., less than 3 mm in diameter and without a distinct margin and hyphae entering the clear zone) (Fig. 2).

Testing of bacterial antagonists in vitro.—Procedures used for testing organisms on agar plates were similar to those described by others (6, 12, 18). Bacterial isolates that produced zones of inhibition in soil dilution plates were streaked on the agar surface along a line through the center of the petri dish. The dishes were kept for 24 hr at 23 ± 1 C, and then a spore suspension of R-72 was atomized onto the agar surface. The media used were SEA, and SEA with autoclaved 1-cm carnation stem pieces embedded in agar (SEA-stem). Zones of inhibition were apparent within 3 to 5 days. On the SEA-stem medium, R-72 mycelium began to grow into the inhibition zone within 3 weeks; however, no zone was reduced in size by more than one-half. Inhibition zones on SEA remained unchanged.

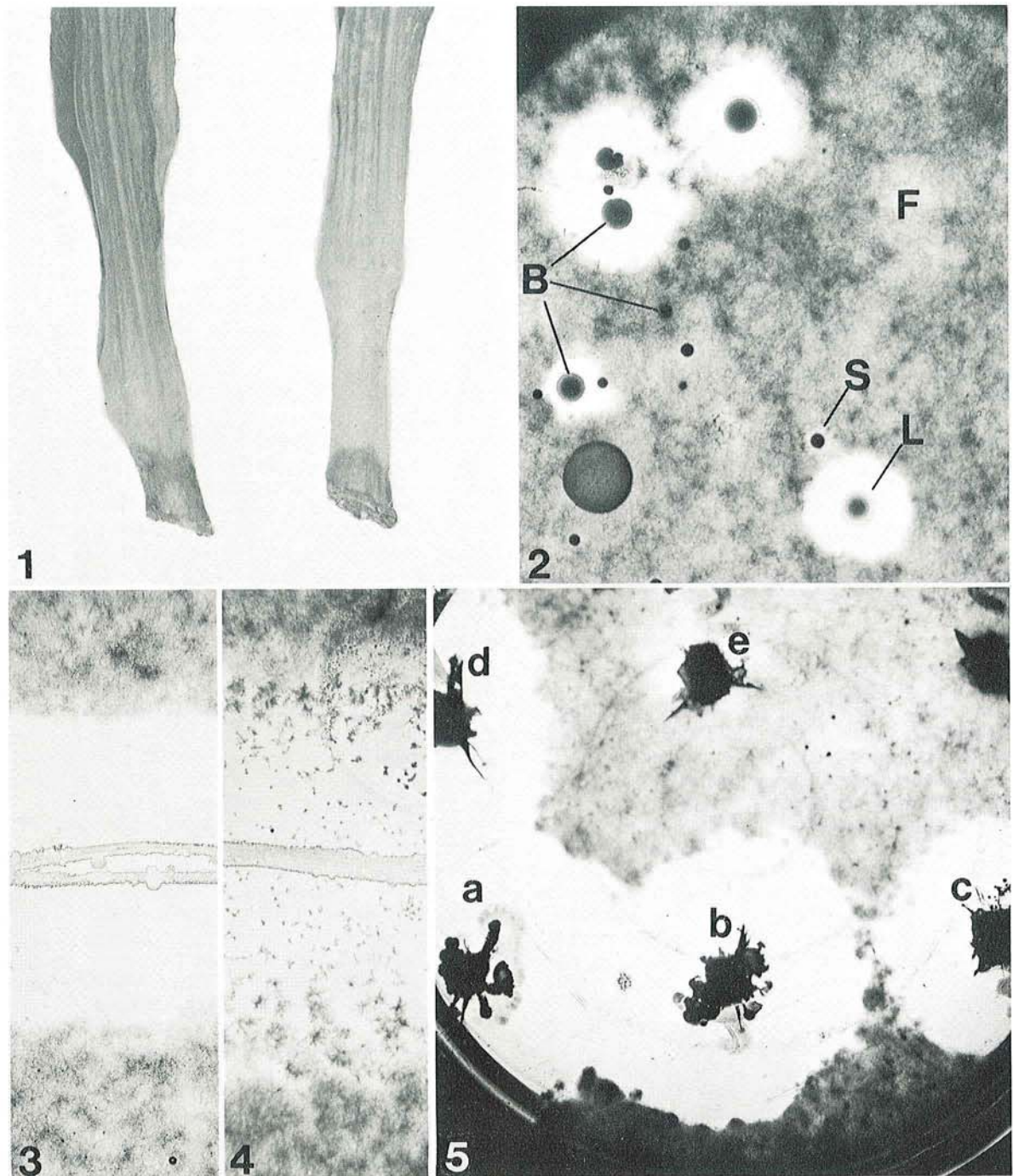


Fig. 1-5. 1) Bases of carnation cuttings inoculated with *Fusarium roseum* 'Culmorum' showing lesions formed. 2) Portion of a soil dilution on soil extract agar + 0.1% peptone + 0.1% dextrose showing mycelium of *F. roseum* 'Culmorum' (F), bacterial colonies (B), zone of inhibition less than 3 mm in diam (S), and zone of inhibition greater than 3 mm in diam (L). 3, 4) Dual culture of isolates of antagonistic bacteria and *F. roseum* 'Culmorum' on soil extract agar showing a zone of inhibition void of mycelial growth (3) and a zone showing abnormal fungus growth (4). 5) Portion of a plate of soil extract agar + 0.1% peptone + 0.1% dextrose with carnation stem sections from a cutting inoculated 30 days previously with antagonistic bacterial isolate A and rooted in a medium infested with *F. roseum* 'Culmorum'. Stem piece (a) is the base of the cutting and the other letters represent consecutive 5-mm stem pieces up the stem. Stem pieces (a) to (d) show zones of inhibition caused by antagonistic bacterial isolate A.

Rating of the inhibition was done 14 days after adding R-72 to the plates, and was based on a 1 to 4 scale. A clear zone, rated 4 (Fig. 3), and a zone containing abnormal growth, rated 3 (Fig. 4), occurred most frequently. Ten bacterial isolates from five soils were selected by this procedure for testing on unrooted carnation cuttings. They were stored in sterile distilled water after growth on NA for two consecutive 48-hr periods.

Carnation cutting tests.—Unrooted cuttings of Improved White Sim were used throughout this study. Stock plants were started from culture-indexed cuttings obtained from Yoder Brothers Inc., Barberton, Ohio. A propagation medium consisting of 1:1 peat-perlite was placed in 5-inch clay pots, steam-treated, then kept under intermittent mist. Various tests were devised to determine if any of the antagonistic bacteria isolated from soil could reduce the number of cuttings that developed symptoms after inoculation with R-72. The bacterial isolates used in each test were grown on NA at 23 ± 1 C for two consecutive 48-hr periods. Inoculum was prepared by suspending the bacteria from a culture in 100 ml of sterile distilled water. Adjustment of the suspensions to a uniform turbidity was effected by means of a Bausch and Lomb Spectronic 20. Carnation cuttings were removed from stock plants, and while held under water were cut with sterile razor blades to a length of 10 to 13 cm. The cut ends of the cuttings were placed in beakers containing 100 ml of a bacterial cell suspension and kept 24 hr in the greenhouse under a light shade provided by two layers of cheesecloth. In tests 1 and 2, described below, three sets of controls were used: (i) a noninoculated control that consisted of 15 unrooted cuttings soaked for 24 hr in sterile distilled water and stuck into the steam-treated propagation medium; (ii) a control that consisted of 15 unrooted cuttings soaked for 24 hr in sterile distilled water and stuck into a steam-treated propagation medium previously infested with macroconidia of R-72; and (iii) a control that consisted of 15 unrooted cuttings soaked for 24 hr in 100 ml of a bacterial cell suspension of the isolate being tested and stuck into the steam-treated propagation medium. This last control served to indicate whether or not the bacteria alone could cause basal stem rot. Fifteen unrooted carnation cuttings were used for each test of each isolate. After treatment of the cuttings was completed, they were divided into three lots of five cuttings each and each lot was placed in a 5-inch pot. Each pot was considered a replicate.

In test 1, 15 cuttings/isolate were treated for 24 hr with the bacterial cell suspension, then placed into a propagation medium infested with the pathogen. The medium was infested by mixing 100 ml of a suspension of R-72 macroconidia in sterile distilled water into the rooting medium in a 5-inch pot 7 days prior to the sticking of the cuttings. In test 2, 15 cuttings/isolate were treated with a bacterial cell suspension for 24 hr and placed for 15 min into a suspension of ca. 10^4 macroconidia of R-72/ml of distilled water. The inoculated cuttings were stuck in a steam-treated rooting medium, five cuttings/5-inch pot. Each pot

was considered a replicate. The rooting media were kept at 22 ± 2 C for both tests. All cuttings were left in the rooting medium for 30 days, then removed and observed for macroscopic lesions (Fig. 1). When all the cuttings had rooted, they were planted in a 1:1:1 soil-peat-perlite mix, and were grown in the greenhouse for an additional 3 months to observe them further for disease development.

The ability of the bacterial antagonists to compete with *F. roseum* in a steam-treated, 1:1 peat-perlite rooting medium supplied with abundant moisture by intermittent misting was determined in test 3. Each 5-inch pot of steam-treated rooting medium was infested with 100 ml of an aqueous suspension of R-72 macroconidia and 100 ml of an aqueous suspension of cells of the selected antagonist. Three pots of rooting medium were prepared for each bacterial isolate and placed under intermittent mist for 7 days; then five freshly cut, untreated, unrooted carnation cuttings were inserted into each pot. Controls consisted of 15 unrooted cuttings stuck in pots of the rooting medium treated with sterile distilled water, and 15 unrooted cuttings stuck in pots of the rooting medium treated with a bacterial cell suspension of the isolate being tested. Each group of 15 cuttings was divided into three lots of five cuttings each and each lot was placed in a 5-inch pot. Each pot was considered a replicate. The rooting medium was held at 23 ± 2 C for 30 days; then cuttings were removed, observed for macroscopic lesions, and rated.

In each of the three tests, the cuttings were rated and assigned a numerical value (disease rating) based on the scale: 1 = no macroscopic basal lesions present and cuttings well rooted; 2 = no macroscopic basal lesions present and cuttings not rooted; 3 = macroscopic basal lesions present. All tests with isolate A were repeated once.

Persistence of antagonistic bacteria in treated carnation stems.—Carnation cuttings were soaked in cell suspensions of selected antagonistic bacteria or in sterile distilled water (controls) and rooted in an R-72 infested medium. Cuttings were selected 1, 10, 20, and 30 days after treatment, surface disinfested with a 1:5 Clorox (5.25% sodium hypochlorite) solution, and sectioned into 5-mm pieces. Alternate sections, after discarding the first one, were placed on SEA + PD or Nash medium (10). A water suspension of R-72 macroconidia was atomized onto the surface of the SEA + PD plates 24 hr later. The plates were incubated at 23 ± 1 C and observed for inhibition zones around the stem pieces after 5 to 7 days. All stem pieces placed on the Nash medium were incubated 2 weeks. Growth characteristics and colony shape were determined for antagonistic bacteria growing from inoculated carnation stem pieces and compared with the original bacterial isolates.

RESULTS.—These tests provided a means of screening potential antagonists in unrooted cuttings (Fig. 6). Soaking the cut ends of unrooted carnation cuttings in cell suspensions of the bacterial antagonists apparently did not affect the rooting of cuttings except in a few cases where brown streaks

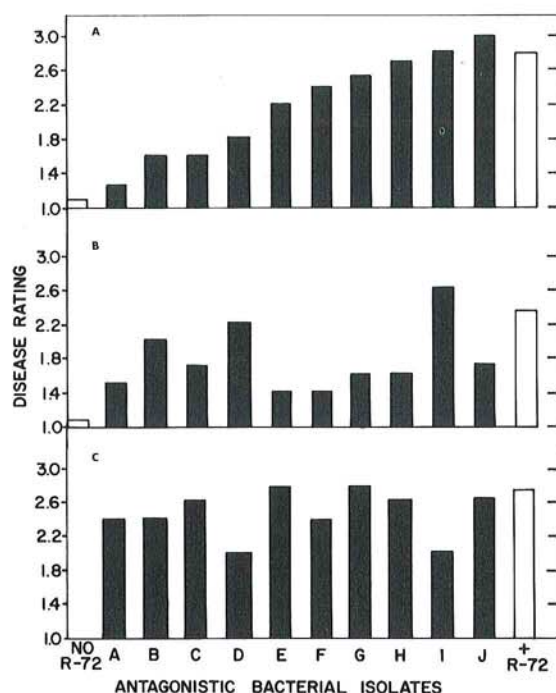


Fig. 6. Results of the propagation of unrooted carnation cuttings. Cuttings were inoculated with antagonistic bacterial isolates by soaking the base of the cuttings in a cell suspension for 24 hr. All readings were made 30 days after the cuttings were placed in the rooting medium. A) Cuttings inoculated with the antagonistic bacterial isolates and placed in a rooting medium infested with *Fusarium roseum* 'Culmorum' (R-72). B) Cuttings inoculated with antagonistic bacterial isolates and immediately placed in a conidial suspension of *F. roseum* 'Culmorum', then into a steam-treated rooting medium. C) Rooting medium simultaneously infested with the selected antagonistic bacterial isolate and *F. roseum* 'Culmorum' 7 days prior to placing cuttings in the rooting medium. The disease rating used was 1 = no macroscopic basal lesions present and cuttings well rooted; 2 = no macroscopic basal lesions present and cuttings not rooted; 3 = macroscopic basal lesions present. No R-72 was the non-inoculated check and +R-72 was the inoculated check.

occurred in the lower stem tissue after 30 days and persisted for the additional 3-month period in which the cuttings were observed. In test 1 (Fig. 6-A), isolate A was effective in reducing the number of inoculated cuttings that exhibited macroscopic lesions, but in test 2 (Fig. 6-B) was less effective. The performance of the other bacterial isolates was erratic (Fig. 6). Cuttings which were free of stem lesions and well rooted at planting time continued to grow and flower normally during a 3-month growing period, whereas cuttings that were poorly rooted or had lesions rotted during this time.

In test 3, none of the bacterial isolates reduced the number of inoculated cuttings that developed macroscopic lesions on the base of the stem. Isolate A, which performed effectively in tests 1 and 2, was ineffective in this test (Fig. 6-C).

Zones of inhibition occurred around stem pieces

sectioned from the lower portion of a carnation stem (Fig. 5). In almost all cases, the zones associated with the inoculated stem pieces were similar to zones produced by the antagonists in dual culture (Fig. 3). Inhibition zones surrounding pieces of cuttings were present 1, 10, 20, and 30 days after treatment, indicating that the antagonistic bacteria were present during the entire propagation period. The height on the cutting at which the antagonistic effect was associated with a stem section varied but averaged 15 to 20 mm from the cutting base and often occurred as high as 30 mm. In all tests involving the pathogen and the bacterial antagonists, the pathogen was isolated from cuttings on Nash medium whether or not there was any reduction in the number of diseased plants.

Bacterial isolate A produced clear, distinct zones of inhibition on agar plates and reduced the number of carnation cuttings with stem rot symptoms. Some preliminary tests indicated that this bacterium has the following characteristics. Cells after 4, 8, 10, and 20 hr growth on NA or PDA are gram-negative. Individual cells are rod-shaped, $1.2-1.6 \times 0.8-1.0 \mu$ (electron microscope measurement), and motile in nutrient broth cultures (phase microscope). Cells negatively stained with 2% phosphotungstic acid in water and observed with an electron microscope have polar flagella (Fig. 7). No growth was observed under anaerobic conditions. These general characteristics place the bacterium in the family Pseudomonadaceae (3). Additional tests showed that growth occurs at 24 and 36 C but not at 45 C. Colonies appear light yellow on nutrient agar and ivory to cream on PDA after



Fig. 7. Electron micrograph showing the polar flagella of bacterial isolate A ($\times 20,000$).

8 days at 21 C. On both media, the colonies are circular with entire edges, smooth glistening surfaces, and convex-to-pulvinate elevations. A pellicle and flocculent growth occurs in still broth culture after 72 hr. The bacterium gives a positive reaction for the cytochrome oxidase test and forms acid but not gas from glucose, sucrose, and lactose in the synthetic medium of Ayers et al. (2). The bacterium does not fluoresce on King's Medium B. Endospores are not formed in cells from 6-day-old cultures stained with a 5% aqueous solution of Malachite Green.

DISCUSSION.—This study indicates that some bacteria which occur naturally in soil, when tested *in vitro*, can restrict the growth of *F. roseum* 'Culmorum'. Selected isolates may reduce the amount of Fusarium stem rot that develops on carnation cuttings in propagation benches. In three tests of the ability of the antagonistic bacterial isolates to reduce the effect of the pathogen on carnation, isolate A (Pseudomonadaceae) (Fig. 6) was the most promising. Nelson (11) reported that initial infection may occur in the propagation bench; therefore, test 1 may represent more accurately the natural means of inoculation of carnation cuttings. In this case, the bacteria were inside the cutting or on the cut surface at the base or in both locations prior to the time the cuttings were placed in the infested rooting medium. The antagonistic bacteria may protect the cut surface at the base of the cutting until it forms a protective layer of callus tissue. The fact that the pathogen could be recovered from symptomless cuttings 4 months after inoculation with an antagonistic bacterium and the fungus pathogen casts some doubt on this possibility. The antagonistic bacteria may also spread throughout the interior of the basal portion of the cutting and, by producing a fungistatic antibiotic, inhibit growth of the fungus. Some bacterial isolates effectively reduced the number of diseased plants, and may provide a possible method of control of the Fusarium stem rot disease of carnation. Test 2 may be more severe than that which occurs naturally, since the viable spores are placed directly on the wounded carnation stem, but this test also indicated that certain isolates were capable of reducing stem rot lesions. Test 3 was an attempt to measure the competitive ability of the pathogen and the bacterial isolates; the interaction between these organisms resulted in little disease reduction.

Carnation cuttings inoculated with bacterial isolates antagonistic against *F. roseum* 'Culmorum' are protected for some time after inoculation. The antagonistic bacteria seem to have an effect similar to fungistasis on the pathogen, since it could be recovered from symptomless carnation cuttings. It is also possible that the bacteria and the antibiotic material are not equally distributed within the carnation stem tissue, thus providing areas of the stem tissue in which the fungus can grow.

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