Interactions Between Bacteria and *Trichoderma hamatum* in Suppression of Rhizoctonia Damping-off in Bark Compost Media

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ABSTRACT

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Eight species of bacterial antagonists, including *Bacillus cereus*, *Enterobacter cloacae*, *Flavobacterium balustinum*, *Janthinobacterium lividum*, *Pseudomonas fluorescens* biovar III, *P. putida*, *P. stutzeri*, and *Xanthomonas maltophilia*, induced suppression to Rhizoctonia damping-off in container media amended with composted hardwood tree bark. Combinations of some of the strains of bacterial antagonists, *E. cloacae* 313, *F. balustinum* 299, *P. fluorescens* biovar V A1, *P. putida* 371, or *P. stutzeri* 280, with *Trichoderma hamatum* 382 were consistently more effective than the fungal isolate alone. Other isolates, for example *P. fluorescens* biovar V A1 and A498, were not effective unless combined with *T. hamatum* 382. Spontaneous rifampicin-resistant mutants of *F. balustinum*, *P. putida*, and *X. maltophilia* colonized both cucumber roots and the conducive container medium to high population levels in irrigated pots. Without irrigation, root colonization was less extensive, and colonization of the container medium was limited to the area surrounding the treated seed. Population levels established were highest in the autoclaved container medium and lowest in the suppressive medium already colonized by a mesophilic microflora. We concluded that suppression of Rhizoctonia damping-off in the naturally suppressive container medium may be due to the activity of a variety of antagonists.

Container media amended with mature bark compost (> 11 wk of composting) are suppressive to Rhizoctonia damping-off (23), and the suppressive effect lasts for at least 2 yr (22). Container media with Canadian sphagnum peat as the sole organic component are conducive (10,22,28), although a source of light sphagnum peat has been described that may be suppressive up to 7 wk after planting (31). The literature on hardwood bark compost for control of Rhizoctonia diseases, until recently, has been limited to container-produced plants. In Florida, however, hardwood bark was used successfully to suppress Rhizoctonia blight of pine seedlings in sandy soils (7).

Suppressiveness of container media amended with composted hardwood tree bark (CHB) is induced by microbes (23). Our previous work with fungi isolated from suppressive and conducive CHB container media has shown that a relationship exists between population levels of certain fungi and suppression of Rhizoctonia damping-off (19). Isolates of *Trichoderma hamatum* (Bonord) Bain. aggr. and *T. harzianum* Rifai, the most abundant fungal taxa isolated from suppressive CHB container media (19), are also the most efficacious fungi in inducing suppression (24).

The role of bacterial antagonists in suppression of Rhizoctonia damping-off in CHB container media has not been examined. This study was initiated to identify bacterial antagonists involved. Furthermore, interactions of the bacterial antagonists with *T. hamatum* were investigated to better understand the mechanism of the suppressive effect. Finally, spontaneous rifampicin-resistant mutants of some of the efficacious bacterial antagonists were selected to follow their population development in CHB container media in the presence and absence of *T. hamatum*.

MATERIALS AND METHODS

Compost and container media. Fresh hammermilled hardwood tree bark (mostly *Quercus* spp.) (Paygro, Inc., South Charleston, OH) was amended with 2 kg of ammonium nitrate and 1.5 kg of urea per cubic meter of bark. Water was added to adjust the moisture level to 55% on a dry weight basis. Compost was prepared in 25-m^3 piles (2 m high) that were turned biweekly. Water was added as needed to maintain a moisture level of approximately 55% (on a dry weight basis). Temperature in the center of the compost pile was monitored routinely.

A CHB container medium was prepared with compost more than 4 mo old, as described previously (22). The compost was mixed with Canadian sphagnum peat and perlite (5:2:3, v/v) to adjust the air-filled pore space at container capacity (10-cm tall column) to 15–20% (calculated from a soil moisture desorption curve). The pH of this container medium ranged from 6.0 to 6.4 and the dry weight bulk density was 0.2 g/cm^3. Slow release fertilizer was added immediately before planting as described previously (24).

Several types of CHB container media, either conducive or suppressive to Rhizoctonia damping-off, were prepared. The naturally suppressive container medium prepared with samples removed from the low temperature edge of compost piles (> 4 mo old) is referred to as CHB_c. A naturally conducive medium (CHB_c) was prepared with samples removed from the high temperature center (> 60 C for five consecutive days after turning) of compost piles produced during summer conditions. Because compost piles with a center temperature of 60 C were not always available, a conducive container medium was prepared by heat treatment (5 days, 60 C) of the CHB_c medium in polyethylene bags in an oven (24). Because the temperature at the edge of the compost pile fluctuates widely with the change of seasons, CHB_c samples were incubated 5 days at 25 C before they were used. These conducive and suppressive media are referred to hereafter as CHB_{60} and

CHB_{25}, respectively. For some experiments, a CHB_e medium was autoclaved 1 hr in 2-L bags on two consecutive days. All heating was done after the mix was formulated.

Bioassay. Suppressiveness to Rhizoctonia damping-off was determined with a radish (*Raphanus sativus* L. cultivar Early Scarlet Globe, 97% germination) bioassay (9,24). Soil inoculum of *R. solani* was produced in a chopped potato soil mixture described by Ko and Hora (16). The inoculum was air dried and screened to yield 1- to 2-mm soil inoculum pieces (22). Container media were infested with 0.5 g of soil inoculum pieces of *R. solani* per liter (equivalent to approximately 2.5 g of air-dried inoculum per kilogram dry weight of container medium). Radish seeds were planted at a mean distance of 1.4 cm from each other in pots containing approximately 400 cm^3 of container medium (32 seeds per 10-cm-diameter pot). Pots were watered and incubated in a growth chamber, as described previously (24). After 7 days, plants were rated according to a disease severity scale in which 1 = symptomless, 2 = diseased but not damped-off, 3 = postemergence damping-off, and 4 = preemergence damping-off (seedlings that did not emerge). Mean disease severity ratings were based on five replicates of 32 seeds per pot. Completely randomized designs were used in all bioassays. Each experiment was performed at least twice. One-way analysis of variance was performed using MINITAB computer program. Separations of means were based on least significant difference (LSD) at $P = 0.05$.

Isolation of bacterial antagonists. Bacteria were isolated from radish roots, cucumber roots (*Cucumis sativus* L. cultivar Straight Eight), or soil inoculum pieces of *R. solani* Kühn incubated in sandwiches in suppressive batches of CHB container media collected from various nurseries, as described previously for isolation of fungal antagonists from this container medium (19). Roots and propagules were rinsed in sterile distilled water and comminuted in a Ten Broeck tissue grinder (0.3 ml of distilled water). Suspensions thus prepared were diluted in buffer (7 g of K_2HPO_4, 3 g of KH_2PO_4, and 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1 L of water) and plated on Difco nutrient agar (NA) and on King's B agar (KB). Plates were incubated 48 hr at 25 C, and colonies were picked at random and purified by streaking and dilution plating on the media from which the bacteria had been isolated. Pure cultures were stored at 4 C on yeast dextrose calcium carbonate agar (32). Cultures were tested for antagonistic activity in bioassays, as described below. Effective antagonists were then stored at −70 C in glycerol-water (27).

Candidate antagonists were cultured 48 hr in Difco nutrient broth (NB) shake cultures (50 ml in 250-ml flask, 25 C), recovered by centrifugation, and resuspended into the dilution buffer described above to population levels of approximately 10^8 colony-forming units (cfu)/ml (verified by dilution plating on NA). These cell suspensions were added to 2 L of conducive CHB_{60} container medium in a polyethylene bag. The inoculum was then distributed evenly by shaking bags vigorously for 30 sec. The final population level of the candidate bacterial antagonist was approximately 10^7 cfu per gram dry weight of container medium. Thereafter, soil inoculum of *R. solani* (1.0 g per 2 L) and slow release fertilizer were added, as described previously (19). The infested CHB_{60} container medium was then distributed evenly in five pots and assayed for suppressiveness as described in the bioassay section. Control treatments were CHB_{60} not treated with potential antagonists and naturally suppressive CHB_{25}, both infested with *R. solani*. Other controls were CHB_{60} and CHB_{25} not infested with *R. solani*. *T. hamatum* 382 (ATCC20765) (24) was added at initial population levels of 10^4 cfu per gram dry weight of CHB_{60} container medium, as described previously (24). Efficacy of antagonist treatments was determined by statistical analyses of mean disease severity indexes established in radish bioassays, as described above. In some experiments efficacy of antagonist treatments was followed by replanting of bioassays (9).

Identification of bacterial antagonists. Bacteria were identified with standard methods and characteristics described by Kreig and Holt (17). Standard diagnostic media were prepared according to methods and formulae given by Fahy and Hayward (5). *Bacillus* spp. and members of Enterobacteriaceae were identified with keys described by Gordon et al (8) and Kelly et al (15), respectively. The taxonomic identity of the isolates was verified by analysis of their fatty acid profiles with an HP5898A microbial identification system using the aerobic library version 1.1, according to procedures specified by the manufacturer (Hewlett-Packard, Avondale, PA).

Production of antifungal substances. The ability of the bacterial antagonists to produce antifungal substances against *R. solani* and *T. hamatum* was determined by paired in vitro assays on potato-dextrose agar (PDA) and KB agar. Bacteria were streaked as a broad band on the agar and allowed to grow for 3 days before an agar plug of either *R. solani* or *T. hamatum* 382 was placed on the plate 40 mm away from the bacteria. Zones of inhibition were measured after 4 days at 25 C.

Isolation of mutants. To determine the distribution of selected antagonists in container media, spontaneous rifampicin-resistant mutants of *Pseudomonas putida* 315, *Flavobacterium balustinum* 299 ATCC53198, and *Xanthomonas maltophilia* 76 ATCC53199 were isolated. Cultures of these three antagonists incubated 24 hr in NB in a shaker at 25 C were seeded (10^9 cells per plate) on KB agar (*P. putida* 315) or NA (*F. balustinum* 299 and *X. maltophilia* 76), both supplemented with 100 μg of rifampicin per milliliter. After 48 hr at 25 C, colonies of spontaneous resistant mutants were streaked onto the same media, without rifampicin. Single colony isolates were stored in 15% glycerol at −70 C. These cultures were then tested for stability of rifampicin resistance, growth rate, and antagonistic activity in the bioassay. Strains not significantly different ($P = 0.05$) in antagonistic activity (*P. putida* 315R, *F. balustinum* 299R, and *X. maltophilia* 76R) from the wild types were selected for further work.

Antagonist enumeration. *P. putida* 315R was enumerated on KB agar supplemented with novobiocin (50 μg/ml), penicillin G (75 units/ml), and cycloheximide (80 μg/ml) (25), to which rifampicin (100 μg/ml) was added. *F. balustinum* 299R and *X. maltophilia* 76R were enumerated on NA supplemented with crystal violet (1.5 μg/ml), neomycin (40 μg/ml), cycloheximide (80 μg/ml), and rifampicin (100 μg/ml). Container media not infested with mutants were included as controls to check for the occurrence of spontaneous rifampicin-resistant mutants with identical colony types and contamination among treatments. *T. hamatum* 382 was enumerated on Elad's medium (4).

Movement of antagonists from seeds into the rhizosphere and nonrhizosphere. Rifampicin-resistant mutants were grown 24 hr in NB. Cells were collected by centrifugation and then resuspended in 10 ml of phosphate buffer. Cucumber seeds were soaked 10 min in one of the bacterial suspensions and then planted in thoroughly watered CHB_{60}, CHB_{25} or autoclaved CHB container medium in Styrofoam pots (9.5 × 17 cm, 14 cm "soil column"). Nonirrigated pots were also watered before planting but were sealed in individual polyethylene bags. Irrigated pots were watered daily. Six days after planting, pots were cut open and cucumber seedlings were carefully removed from the container medium. Particles of the CHB container media were removed from taproots. In addition, lateral roots were cut off because final population levels were based on root length (centimeter). The taproot was then cut into five 2-cm segments. Each segment was ground in 1 ml of diluent (0.4M NaCl and 0.05M $MgSO_4$) with a mortar and pestle. Mutant populations were then enumerated by 10-fold serial dilutions (triplicate series) on selective media. Three plants were used per treatment and each plant was considered as a replicate. The entire experiment was repeated once.

A separate set of pots was used to determine colonization of nonrhizosphere container media ("bulk soil") by bacterial antagonists. After the plant had been removed, 10-g container medium samples were taken from the top, middle, and bottom of the pot. The samples were shaken in 50 ml of diluent for 15 min, and mutants were enumerated on selective media as described above. The experiment was performed twice.

Survival of antagonists in container media. *F. balustinum* 299R, *P. putida* 315R, and *X. maltophilia* 76R were added to CHB_{60}, CHB_{25}, and autoclaved CHB at 10^6 cfu per gram dry weight of container medium. *T. hamatum* 382 was added at 10^4 cfu per gram

dry weight. One cucumber seed was planted in each pot. Pots were watered daily. Samples were taken at 0 and 24 hr and at 7-day intervals thereafter, for a period of 28 days. All roots were carefully removed from container media. The medium from one pot was then shaken vigorously (30 sec) in a polyethylene bag (three replications per treatment), and mutant population levels in 10-g samples were then determined as described above. The experiment was repeated once.

In two other experiments, survival of antagonists in CHB_e and CHB_c container media was evaluated. Efficacy of the antagonist treatments in suppression of Rhizoctonia damping-off in these container media was evaluated as well.

RESULTS

Efficacy of antagonists. A total of 562 bacterial isolates obtained by dilution plating from cucumber and radish roots and from soil inoculum pieces of *R. solani*, retrieved from naturally suppressive CHB, were screened for their ability to induce suppression to Rhizoctonia damping-off in the radish bioassay in heat-treated bark compost (CHB_{60}) medium. The most efficacious isolates were identified to species and are listed in Table 1. The taxonomic identity of the strains, with exception of *F. balustinum* 299 that was not in the HP5898A library, was verified by analysis of their fatty acid profiles.

The disease severity rating of the radish bioassay in CHB_{60} not treated with antagonists was 3.3. Ten of 13 bacterial strains significantly ($P = 0.05$) reduced damping-off when added as single antagonist treatments. *T. hamatum* 382, as a single antagonist treatment, reduced disease to a severity rating of 2.8. Six of the bacterial strains listed (*E. cloacae* 313, *J. lividum* 275, *P. fluorescens* biovar V A1 and A498, *P. putida* 315, and *P. stutzeri* 280), when combined with *T. hamatum* 382, significantly ($P = 0.05$) reduced the disease severity over that induced by the bacteria alone. Five bacterial strains (*E. cloacae* 313, *F. balustinum* 299, *P. fluorescens* biovar V A1, *P. putida* 371, and *P. stutzeri* 280), significantly ($P = 0.05$) enhanced efficacy of *T. hamatum* 382. Three bacterial strains, i.e., *P. fluorescens* A1 and A498 and *P. putida* 315, had a significant effect only if added in combination with *T. hamatum* 382. None of the bacterial antagonists reduced the efficacy of *T. hamatum* 382.

Long-term effects of *T. hamatum* 382 in combination with *F. balustinum* 299 were followed by replanting of bioassays over a 5-wk period. After five replantings, the disease severity rating of the CHB_{25} and the CHB_{60} media were 2.2 and 3.0, respectively. The disease severity value for the CHB_{60} medium amended with *T. hamatum* 382 and *F. balustinum* 299 was 2.2 ($LSD_{0.05} = 0.3$). The combination antagonist treatment, therefore, significantly ($P = 0.01$) reduced disease severity over that present in the unamended CHB medium and was not different from the naturally suppressive CHB_{25} medium.

The efficacy of *T. hamatum* 382 and of *F. balustinum* 299R in the container media amended with compost from the edge and center of the pile (CHB_e and CHB_c) was followed in two experiments. Results of both experiments were similar. Mean disease severity values, based on the two experiments, for both the CHB_e and the CHB_c container medium not infested with *R. solani* were 1.3. Values in *R. solani*-infested CHB_e and CHB_c container media were 2.7 and 3.7, respectively. Mean disease severity values for the *T. hamatum* 382 treatment, the *F. balustinum* 299R treatment and the combination treatment of these antagonists in CHB_c were 3.0, 3.5, and 2.5, respectively ($LSD_{0.05} = 0.4$). The combination antagonist treatment, therefore, again was significantly ($P = 0.05$) more effective than the single antagonist treatment. The treatments had no effect in CHB_e that already was suppressive.

Production of antifungal substances. Of 13 bacterial strains, only four produced antifungal substances against *R. solani* in paired in vitro assays. Two fluorescent pseudomonads (A1 and A91) produced inhibition zones on both PDA and KB agar. *E. cloacae* 313 produced inhibition zones on PDA only, whereas *X. maltophilia* 76 produced inhibition zones on KB agar only (Table 2).

Only three bacterial strains produced zones of inhibition against *T. hamatum* 382. Two of these, *E. cloacae* 313 and *P. fluorescens* biovar III A91, produced inhibition zones on PDA only, while the third one, *P. stutzeri* 280, produced inhibition zones on KB agar only.

Colonization of cucumber roots by bacterial antagonists. The ability of *F. balustinum* 299R, *P. putida* 315R, and *X. maltophilia* 76R to colonize cucumber roots in CHB_{60}, CHB_{25}, and autoclaved CHB container media is presented in Table 3. In the autoclaved container medium, all three mutants consistently colonized the entire cucumber root system from the treated seed, irrespective of irrigation treatment. Furthermore, high population levels were

TABLE 1. Suppression of Rhizoctonia damping-off of radish induced by bacterial antagonists alone and in combination with *Trichoderma hamatum* in a container medium amended with composted hardwood tree bark (CHB)

	Disease severity rating[a]	
Bacterial antagonist[b]	Bacterial antagonist alone	Bacterial antagonist with *T. hamatum*
None	3.3	2.8
Bacillus cereus 106	2.7	2.6
Enterobacter cloacae 127	2.8	2.5
E. cloacae 313	2.7	2.3
Flavobacterium balustinum 299 (ATCC 53198)	2.4	2.1
Janthinobacterium lividum 275	2.9	2.5
Pseudomonas fluorescens biovar III A91	2.6	2.5
P. fluorescens biovar V A1	3.0	2.4
P. fluorescens biovar V A498	3.2	2.7
P. putida 305	2.8	2.5
P. putida 315	3.1	2.6
P. putida 371	2.2	2.3
P. stutzeri 280	2.5	2.1
Xanthomonas maltophilia 76 (ATCC 53199)	2.4	2.5
$LSD_{0.05}$	0.3	

[a] Inoculated with 0.5 g of soil inoculum of *Rhizoctonia solani* per liter of container medium. Mean disease severity rating determined 7 days after incubation at 26 C from five pots planted with 32 radish seeds each: 1 = symptomless; 2 = diseased but not damped-off; 3 = postemergence damping-off; and 4 = preemergence damping-off. Mean disease severity in the container medium not infested with *R. solani* was 1.2.

[b] Bacterial antagonists were added to the heated bark compost medium (CHB_{60}) at initial population levels of 10^6 cfu/g dry weight. *T. hamatum* was added at 10^4 cfu/g dry weight.

TABLE 2. Production of antifungal substances by bacterial antagonist against *Rhizoctonia solani* and *Trichoderma hamatum* on potato dextrose agar (PDA) and King's agar (KB)

	Rhizoctonia solani		*Trichoderma hamatum*	
Bacterial antagonist	PDA	KB	PDA	KB
Bacillus cereus 106	–[a]	–	–	–
Enterobacter cloacae 127	–	–	–	–
E. cloacae 313	+++	–	++	–
Flavobacterium balustinum 299 (ATCC 53198)	–	–	–	–
Janthinobacterium lividum 275	–	–	–	–
Pseudomonas fluorescens biovar III A91	+	+++	+	–
P. fluorescens biovar V A1	+	+	–	–
P. fluorescens biovar V A498	–	–	–	–
P. putida 305	–	–	–	–
P. putida 315	–	–	–	–
P. putida 371	–	–	–	–
P. stutzeri 280	–	–	–	++
Xanthomonas maltophilia 76 (ATCC 53199)	–	++	–	–

[a] –, +, ++, and +++ represent 0-, 5-, 10-, and 15-mm-wide zones of inhibition in paired petri dish assays.

established on root tips in irrigated pots in the autoclaved medium. In the conducive CHB_{60} container medium, root colonization was less extensive, and in nonirrigated pots, population levels on root tips were low. Irrigation, again, increased population levels on root tips. Finally, in the suppressive CHB_{25} container medium, only the 4-cm root area nearest the seed was colonized to a significant extent (100 cfu per centimeter of root). Irrigation did not result in colonization of root tips by the mutants.

Colonization of container media by bacterial antagonists. *F. balustinum* 299R and *X. maltophilia* 76R did not establish high population levels at a distance greater than 2 cm from the infested seed in media that were not irrigated (Table 4). Without irrigation, *P. putida* 315R also did not colonize the CHB_{60} or the CHB_{25} medium, but it did establish high population levels in the autoclaved CHB medium, up to a distance of 6–8 cm from the treated seed.

All three mutants established highest population levels throughout the irrigated, autoclaved container medium. Lower population levels were established in the conducive CHB_{60} container medium. Finally, in the suppressive CHB_{25} container medium, irrigation established low population levels throughout the pots. Similar results were obtained in a second experiment with these mutants.

Survival of antagonists in the container medium. Population development of *F. balustinum* 299R, *P. putida* 315R, *X. maltophilia* 76R, and *T. hamatum* 382 during 28 days after planting of radish seeds, is presented in Figures 1 and 2. In the autoclaved container medium, the bacterial antagonists established highest population levels within 7 days after planting. Thereafter, populations declined to 10^6–10^7 cfu per gram dry weight. In the conducive CHB_{60} container medium, lower population densities developed, but all remained at levels above 10^6 cfu per gram dry weight. In the suppressive CHB_{25} container medium, populations did not increase but declined to 10^5 cfu per gram dry weight or less. In the CHB_{60} treatment, to which the mutant bacterial antagonists and *T. hamatum* 382 were added

TABLE 3. Colonization of cucumber roots by bacterial antagonists introduced with inoculated seeds in a bark compost container medium (CHB) pretreated with various temperature regimes

Vertical distance from seed (cm)	Log cfu/cm root[a]					
	Autoclaved CHB		Heated medium (CHB_{60})		Control medium (CHB_{25})	
	Not irrigated	Irrigated	Not irrigated	Irrigated	Not irrigated	Irrigated
Flavobacterium balustinum 299R						
0–2	6.98 ± 0.05	6.89 ± 0.18	5.98 ± 0.16	5.63 ± 0.16	5.64 ± 0.04	4.32 ± 0.16
2–4	5.83 ± 0.19	6.15 ± 0.23	3.44 ± 0.52	5.20 ± 0.47	3.12 ± 0.21	2.55 ± 0.58
4–6	4.56 ± 0.28	5.97 ± 0.27	2.70 ± 0.68	4.12 ± 0.33	2.33 ± 0.21	ND[b]
6–8	4.31 ± 0.57	5.87 ± 0.20	2.46 ± 0.59	3.89 ± 0.24	ND	ND
8–10	4.43 ± 0.03	5.70 ± 0.15	1.88 ± 0.48	4.15 ± 0.39	ND	ND
Pseudomonas putida 315R						
0–2	6.49 ± 0.02	6.42 ± 0.09	5.07 ± 0.34	6.30 ± 0.16	4.70 ± 0.28	3.36 ± 0.49
2–4	5.82 ± 0.17	5.56 ± 0.12	3.30 ± 0.23	4.08 ± 0.58	2.79 ± 0.80	2.07 ± 0.34
4–6	5.37 ± 0.13	5.36 ± 0.13	3.38 ± 0.18	3.78 ± 0.36	2.20 ± 0.80	1.77 ± 0.20
6–8	4.61 ± 0.18	5.32 ± 0.06	2.72 ± 0.31	3.92 ± 0.74	1.86 ± 0.46	ND
8–10	3.70 ± 0.14	5.33 ± 0.17	2.13 ± 0.50	3.77 ± 0.47	ND	ND
Xanthomonas maltophilia 76R						
0–2	5.84 ± 0.56	6.97 ± 0.08	4.74 ± 0.29	6.47 ± 0.14	3.85 ± 0.62	4.30 ± 0.30
2–4	3.63 ± 0.65	6.16 ± 0.10	2.96 ± 0.44	5.00 ± 0.23	2.08 ± 0.31	3.38 ± 0.52
4–6	3.22 ± 0.91	6.00 ± 0.06	2.22 ± 0.45	4.60 ± 0.29	ND	2.09 ± 0.35
6–8	3.58 ± 0.36	5.84 ± 0.08	1.79 ± 0.23	4.20 ± 0.36	ND	1.86 ± 0.32
8–10	3.82 ± 0.51	5.67 ± 0.27	1.69 ± 0.29	3.36 ± 0.75	ND	ND

[a] Determined 6 days after planting by dilution plating on selective media. Values are means of three replicates followed by the standard error. Inoculum populations on seeds at the time of planting were 2.7×10^7, 1.9×10^7, and 5.5×10^8 cfu per seed for *F. balustinum* 299R, *P. putida* 315R, and *X. maltophilia* 76R, respectively.

[b] Not detectable (< 50 cfu/cm of root).

TABLE 4. Colonization of the nonshizosphere bark compost container medium (CHB) by bacterial antagonists introduced with inoculated seeds in a container medium pretreated with various temperature regimes

Depth in container medium (cm)	Log cfu/g dry weight container medium[a]					
	Autoclaved CHB		Heated medium (CHB_{60})		Control medium (CHB_{25})	
	Not irrigated	Irrigated	Not irrigated	Irrigated	Not irrigated	Irrigated
Flavobacterium balustinum 299R						
0–2	8.17 ± 0.31	9.07 ± 0.18	5.66 ± 0.44	7.48 ± 0.03	3.91 ± 0.23	5.37 ± 0.19
6–8	1.70 ± 0.30	8.78 ± 0.20	ND	6.44 ± 0.14	ND	3.07 ± 0.30
12–14	1.96 ± 0.56	8.81 ± 0.12	ND	6.17 ± 0.11	ND	2.95 ± 0.06
Pseudomonas putida 315R						
0–2	8.68 ± 0.20	8.71 ± 0.05	5.32 ± 0.28	6.69 ± 0.15	3.80 ± 0.28	4.43 ± 0.07
6–8	7.17 ± 0.42	8.22 ± 0.06	ND	6.04 ± 0.15	ND	3.06 ± 0.21
12–14	2.00 ± 0.46	7.50 ± 0.34	ND	6.06 ± 0.10	ND	2.84 ± 0.12
Xanthomonas maltophilia 76R						
0–2	6.70 ± 0.45	9.70 ± 0.03	5.08 ± 0.39	7.49 ± 0.07	4.55 ± 0.63	4.74 ± 0.06
6–8	ND[b]	9.53 ± 0.12	ND	7.07 ± 0.06	ND	3.61 ± 0.09
12–14	ND	9.42 ± 0.05	ND	6.67 ± 0.12	ND	3.07 ± 0.03

[a] Determined 7 days after planting by dilution plating on selective media. Values are means of three replicates followed by the standard error. Mean inoculum populations on seeds at the time of planting were 2.7×10^7, 1.9×10^7, and 5.5×10^8 cfu per seed for *F. balustinum* 299R, *P. putida* 315R, and *X. maltophilia* 76R, respectively.

[b] Not detectable (< 50 cfu/g dry weight of container medium).

together, bacterial population development was not different from that in the absence of *T. hamatum* 382.

In the autoclaved container medium, population development of *T. hamatum* 382 reached the highest level after 14 days and did not decline thereafter (Fig. 2). Lower populations were established in the conducive CHB_{60} container medium. In the suppressive CHB_{25} container medium, population levels did not change. One week after the addition of *F. balustinum* 299R in combination with *T. hamatum* 382, a slightly higher population level of *T. hamatum* 382 was present as compared with the treatment where only *T. hamatum* 382 was added. Thereafter, the presence of *F. balustinum* 299R had no effect on *T. hamatum* 382 population development.

Survival of antagonists in natural compost. Population development of *F. balustinum* 299R and of *T. hamatum* 382 as single and as combination antagonist treatments in container media prepared with compost removed from the suppressive edge (CHB_e) and conducive center (CHB_c) of the compost pile is presented in Figure 3. High population levels (approximately 10^8 cfu per gram dry weight) of *F. balustinum* 299R were established within 2 days after infestation of the conducive CHB_c container medium. Thereafter, populations declined to levels found previously in the CHB_{60} container medium (Fig. 1A). In the suppressive CHB_e container medium, high populations also developed, which were followed by a decline. *T. hamatum* 382 on the other hand, continued to increase in population level in both the CHB_c and CHB_e container media. *T. hamatum* 382 again did not affect the population development of *F. balustinum* 299R. However, *F. balustinum* 299R increased the population development of *T. hamatum*, as observed earlier in the CHB_{60} container medium (Figs. 2 and 3). Similar results on survival of antagonists were obtained in a second experiment with natural compost.

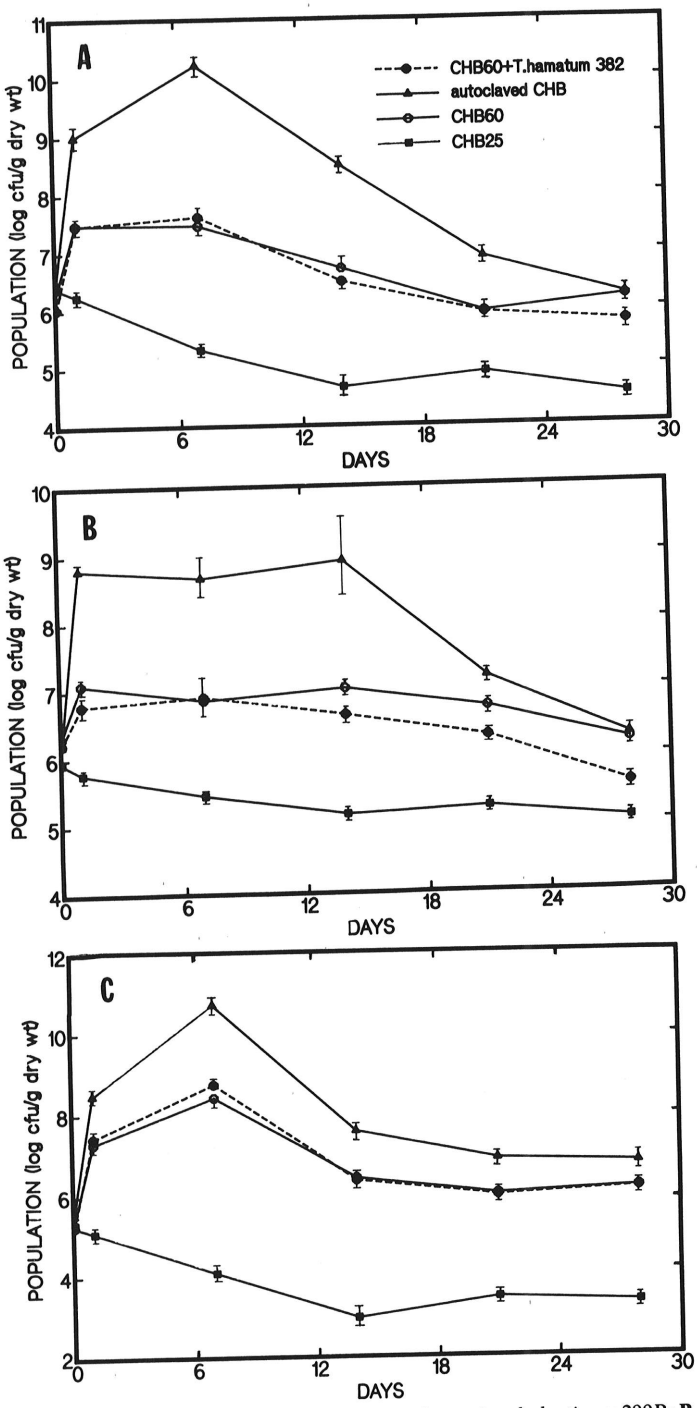

Fig. 1. Population development of **A,** *Flavobacterium balustinum* 299R; **B,** *Pseudomonas putida* 315R; and **C,** *Xanthomonas maltophilia* 76R in an irrigated bark compost container medium (CHB) treated with various temperature regimes and planted with cucumber. Container media were autoclaved CHB; heat-treated at 60 C for 5 days (CHB_{60}); or incubated at 25 C for 5 days (CHB_{25}). Each medium was infested with 10^6 cfu bacterial antagonist per gram dry weight. A fourth comparison was the CHB_{60} container medium infested with the same level of the bacterial antagonist and with *T. hamatum* 382 at 10^4 cfu per gram dry weight. Vertical bars indicate standard error.

DISCUSSION

Several bacterial strains were identified that induced suppression to Rhizoctonia damping-off in conducive container media (Table 1). Efficacy of some was enhanced if introduced in combination with *T. hamatum* 382. Others were effective only if combined with this fungal isolate. Several of the bacterial species listed, i.e., *F. balustinum*, *J. lividum*, *P. stutzeri*, and *X. maltophilia*, have not been reported previously as biocontrol agents for soilborne plant pathogens. *E. cloacae* (1), *P. putida* (26), and *P. fluorescens* (3) are reported most frequently as beneficial rhizobacteria. We chose to explore population development of strains *F. balustinum* 299, *P. putida* 315, and *X. maltophilia* 76 in detail in this system, because two of these have not been examined as antagonists of plant pathogens in the rhizosphere previously. *P. putida* 315 was included for comparison.

Population levels of all three rifampicin-resistant mutants developed to significantly higher levels in the autoclaved medium (approximately $10^{9\text{-}10}$ cfu per gram dry weight), regardless of whether they were introduced as seed treatments (Tables 3 and 4) or directly into the media at planting (Figs. 1 and 3). This shows

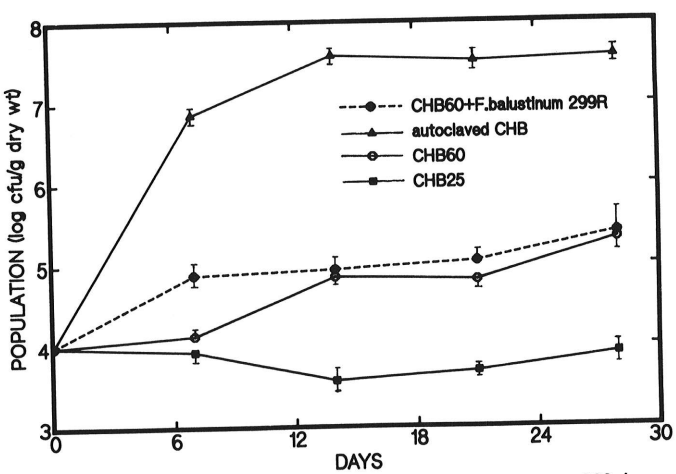

Fig. 2. Population development of *Trichoderma hamatum* 382 in an irrigated bark compost container medium (CHB) treated with various temperature regimes and planted with cucumber. Container media were autoclaved CHB; heat-treated at 60 C for 5 days (CHB_{60}); or incubated at 25 C for 5 days (CHB_{25}). Each medium was infested with 10^4 cfu per gram dry weight of *T. hamatum* 382. A fourth comparison was the CHB_{60} container medium infested with the same level of *T. hamatum* 382 and with *Flavobacterium balustinum* 299R at 10^6 cfu per gram dry weight. Vertical bars indicate standard error.

that the conducive high temperature compost-amended medium (CHB_c) and the heat-treated medium (CHB_{60}) were not a total biological vacuum. In both media, after an initial population increase, levels of the bacterial mutants declined to approximately similar levels after 28 days (10^{6-7} cfu per gram dry weight). The population level of *T. hamatum* 382, on the other hand, increased with time in both types of conducive media (Figs. 2 and 3) but did not decline thereafter. Interestingly, suppressiveness of the CHB_{60} medium amended with *T. hamatum* 382 and *F. balustinum* 299 did not decrease with time. Possibly the effect of the decreasing population level of *F. balustinum* 299 on suppression of Rhizoctonia damping-off was offset by the increase in the population level of *T. hamatum* 382 during the second and third week after their addition to the medium. The decline in population level of all three bacterial mutants occurred, however, even in the absence of *T. hamatum* 382 (Fig. 1). This suggests that other microorganisms could be involved as well.

Population development of *F. balustinum* 299R in the naturally suppressive CHB_e medium (Fig. 3) was different from that in CHB_{25} medium (Fig. 1A). The bacteria developed to high levels in the CHB_e medium but not in the CHB_{25} medium. Similar results were obtained with other spontaneous rifampicin-resistant mutants (P. C. Fahy and H. A. J. Hoitink, *unpublished*). The batches of CHB_e used in this work had been prepared under summer conditions when temperatures of 40 C may prevail in the surface layer of compost piles (18). Apparently, at the higher temperature a greater biological vacuum was present. The bacterial species diversity (29) and the activity of the microbiota (2,20) in various temperature zones of compost piles vary. For example, at temperatures > 60 C few bacterial species survive. Both microbial activity and species diversity increase at lower temperatures. The mushroom industry takes advantage of this. Spawn is introduced most effectively if the temperature of the compost is maintained at 55–57 C before inoculation (6). Concise temperature guidelines for recolonization of compost with beneficial microorganisms such as *F. balustinum* 299 and *T. hamatum* 382 remain to be developed.

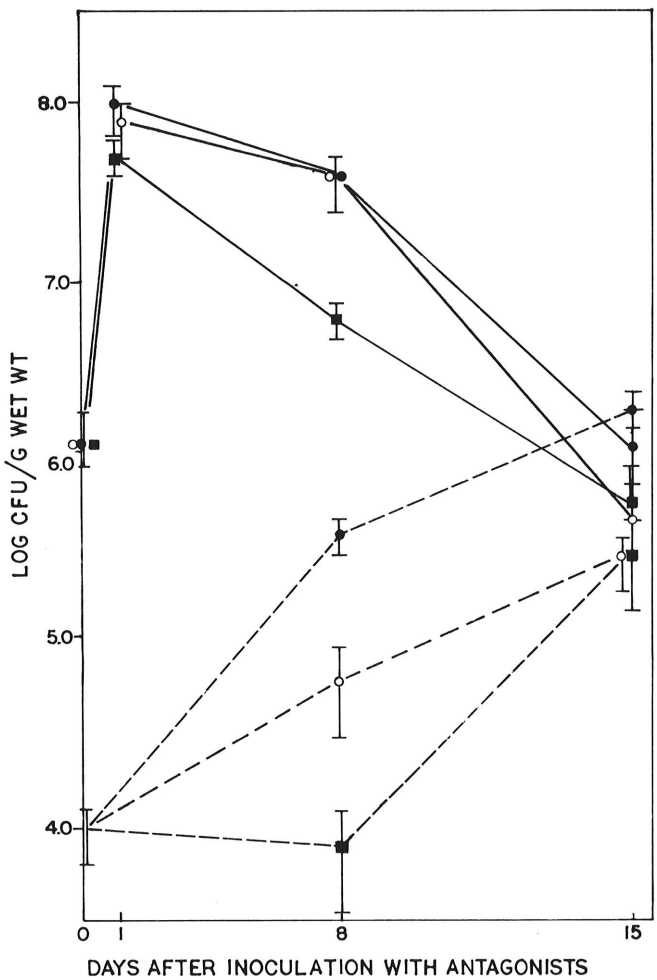

Fig 3. Population development of *Flavobacterium balustinum* 299R (solid line) and *Trichoderma hamatum* 382 (dashed line) in a container medium amended with natural compost and planted with cucumber. *F. balustinum* and *T. hamatum* were added individually to container media prepared with edge bark compost (CHB_e, closed square) or center bark compost (CHB_c, open circle) or together to CHB_c (closed circle). Vertical bars indicate standard error.

It is of interest that few of the bacterial strains that effectively induced suppression produced zones of inhibition in paired agar tests with either *R. solani* or *T. hamatum* 382 (Table 2). *F. balustinum* 299, *P. putida* 315, *X. maltophilia* 76, and their respective rifampicin-resistant mutants, did not produce zones of inhibition in paired assays with *T. hamatum* 382 on KB agar or PDA. This may account for the lack of a negative impact of *F. balustinum* 299R on population development of this *Trichoderma* isolate or its ability to induce suppression in the conducive container media. Some evidence was obtained, in fact, that suggested that *F. balustinum* 299R enhanced population development of *T. hamatum* 382 for a short period after its addition to these container media (Figs. 2 and 3). Combination treatments with beneficial microorganisms, if applied together on seed, may cancel out individual effects, however. For example, a *Pseudomonas* strain antagonistic to *T. hamatum*, negates the efficacy of this fungal isolate against Pythium damping-off (12). Interestingly, none of the combination treatments listed in Table 1 resulted in a significant ($P = 0.05$) loss in efficacy of either isolate applied alone. At least two other combination fungal-bacterial treatments have been proposed for suppression of Rhizoctonia diseases (14,21).

The relative contribution of the bacterial antagonists to the overall suppressive effect of bark compost to Rhizoctonia damping-off remains to be determined. On the other hand, the importance of fungal antagonists, including *Trichoderma* spp., in this system has been explored (19,23,24). In these container media activity of *T. hamatum* 382 varies with maturity of the compost, and this antagonist is most efficacious in container media amended with mature (> 4 mo old) bark compost (24). The effect of compost maturity on activity of bacterial antagonists, the origin of the antagonists and just how bacterial antagonists survive the composting process, which affect consistency of suppressiveness, remain to be determined also.

Natural recolonization of compost can result in chance colonization with deleterious microorganisms (30) or even pathogens. For example, if adequate precautions are not taken, *Salmonella* may recolonize composted municipal sludge after peak heating, but before adequate stabilization has occurred. To avoid this problem, the curing process is monitored at such composting plants (13). Present trends are to produce composts from municipal sludges in closed and more efficient systems that effectively destroy fecal pathogens through heat treatment (18). Unfortunately in this process, beneficial microorganisms are also destroyed. These composts are conducive to Rhizoctonia damping-off even after 2 mo of curing (11). In the future, therefore, controlled inoculation with antagonists, either as pure cultures or as mixtures (33), may become necessary. The Ohio State University has a patent (#4642131) for the production of composts that are predictably suppressive to Rhizoctonia damping-off through introduction of specific antagonists.

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