Development of In Vivo Assays for Prescreening Antagonists of *Rhizoctonia solani* on Cotton

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ABSTRACT

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Presereens of bacteria for biological control of soilborne diseases traditionally eonsist of tests for antibiosis in vitro. Two in vivo assays were developed as possible alternative prescreens for selecting candidate biological control agents for preemergenee and postemergence damping-off diseases of cotton caused by *Rhizoctonia solani*. In the "radicle assay," radicles from surface-disinfested sced were inoculated with *R. solani*. In the "hypocotyl assay," excised surface-disinfested hypocotyls were inoculated with the pathogen. Inoculated radicles and hypocotyls were rated daily for symptom development for the next 6–7 days using a scale of 0–7 (0 = no symptoms; 7 = complete necrosis). The effects of growth medium for the fungal inoculum, form of inoculum, and age of host tissue on the development of necrosis were determined for both assays. The assays were "optimized" by selecting those experimental parameters that allowed a progressive development of symptoms. When tested in the optimized radicle and hypocotyl assays, the previously reported biological control rhizobacteria strains Pf-5 (*Pseudomonas fluorescens*) and GBO-3 (*Bacillus subtilis*) significantly reduced symptom development. Pf-5 populations increased during the evaluation time from 0.5 to 4 log units on radicles and from 3 to 7 log units on hypocotyls, depending on the inoculum density. Populations of GBO-3 on radicles increased 3 log units with low inoculum density but decreased 1.4 log units with high inoculum density, whereas, on hypocotyls, populations increased 1.4 log units with low inoculum density and decreased 3.5 log units with high inoculum density.

Despite increasing research emphasis on biological control of soilborne pathogens, relatively few effieacious biological control agents are available (13,17). Reasons for this relative lack of success include variability in performance and limited modes of action for speeific efficacious strains. Selection of larger numbers of efficacious strains would increase the probability of discovering different underlying modes of action. However, in order to select multiple strains, hundreds or thousands of candidate biological control agents must be screened in replicated disease assays that are often laborious and frequently require controlled-environment facilities.

The use of a relatively rapid prescreen for evaluating interference by candidate biological control agents in disease development would greatly increase the number of strains that could be tested. Past strategies for prescreens have concentrated on antibiosis in vitro, using the definition of Fravel (5), where antibiosis is "antagonism mediated by specifie or nonspecific metabolites of microbial origin." Determining that antibiosis in vitro is correlated with antibiosis in vivo is difficult (5,16,22,24), even when using analysis of mutants lacking antibiosis (20).

Selecting agents based on prescreens for antibiosis in vitro has led to the discovery of many biological control agents (27), and some of these agents have been shown through mutational analysis to provide control through antibiosis in vivo (8,9,25). The use of antibiosis in vitro restricts the discovery of underlying mechanisms for biologieal eontrol to antibiosis through production of antibiotics, toxic metabolites, or siderophores. This approaeh would not seleet agents that provide control by competition, parasitism, avirulenee, or cross protection/induced resistance (14).

Reeent bioeontrol researeh with foliar diseases has used in vivo presereens in whieh candidate biocontrol agents are evaluated for interference on leaf surfaees (18,23). Randhawa and Civerolo (18) monitored reductions in epiphytic population densities of *Xanthomonas campestris* pv. *pruni* in response to leaf colonization by epiphytic bacteria. Spurr (23) showed that nonpathogenic *Alternaria alternata* (Fr.:Fr.) Keissl. reduced disease indices of *A. alternata* on detached tobacco leaves. This approach overcomes some of the limitations of in vitro prescreens while maintaining the ability to screen rapidly large numbers of strains.

Randhawa and Schaad (19) developed a seedling bioassay chamber (SBA) for in vivo assessment of root colonization and antagonism to soilborne pathogens. In this system, seedlings germinate in paper pouches, and roots grow into a selective agar medium used for detecting root colonization. Although it allows for screening of candidate biological control agents in the presence of the host, the SBA is time-consuming to prepare, and growth of biocontrol agents on nutrient-rich selective media may affect their antagonistic potential toward pathogens. No disease index system was used in the SBA study, and, therefore, biocontrol potential was not based on reduction in symptom development.

The overall goal of the following study was to develop rapid in vivo prescreens, with disease indices, for selecting potential biological control agents of preemergence and postemergence damping-off of cotton caused by *Rhizoctonia solani* Kühn. The prescreen assays were optimized by determining the relationship between disease development and the form of *R. solani* inoculant used, the media used to grow the pathogen, and the age of the host tissue. The potential of the optimized assays for selecting candidate biological eontrol agents was tested using two known biological control agents of *R. solani* on cotton.

MATERIALS AND METHODS

Microbial strains. The *R. solani* isolate (AG-4) was obtained from Gustafson, Inc., Dallas, TX, and was originally isolated from cotton. *R. solani* was maintained in autoclaved soil at room temperature between experiments. Before use in the experiments, infested soil was transferred to potato-dextrose agar (PDA) (Difeo, Detroit, MI) and ineubated for 96 h at 25 C.

Pseudomonas fluorescens strain Pf-5 was obtained from Charles Howell, USDA, College Station, TX, and was previously reported as a biological control agent of *Pythium ultimum* Trow (7) and *R. solani* (6) on cotton. *Bacillus subtilis* strain GBO-3 was obtained from Gustafson, Inc., Dallas, TX. GBO-3 originated as a seleetion of *B. subtilis* strain A-13 (13) and was reported to provide biological control of *R. solani* on cotton (1). Bacteria were stored at −80 C in 20% glycerol-amended tryptie soy broth (TSB) (Difco, Detroit, MI) between experiments.

For use in experiments, bacteria were streaked onto tryptic soy agar (TSA) (Difco, Detroit, MI) and checked for purity after incubation for 48 h at 28 C. Single colonies were inoculated into

15 ml TSB incubated with shaking at 150 rpm at 28 C for 24 (strain Pf-5) or 48 h (strain GBO-3). Bacterial suspensions were centrifuged for 5 min at 6,000 rpm, and the cells were rinsed with 20 mM phosphate buffer pH 7.0 (PB). Cells were resuspended in PB, resulting in a population of log 9 colony forming units per milliliter (cfu/ml) as determined by serial dilution and plating on TSA.

Spontaneous rifampicin-resistant mutants were selected (Pf-5 rif and GBO-3 rif) using procedures described previously (10). These mutants were used in experiments (described below) to monitor bacterial population dynamics on radicles and hypocotyls during the assay.

Escherichia coli strain K-12 was obtained from Dorothy Geiger, Department of Botany and Microbiology at Auburn University. The strain was maintained in long-term storage at −80 C and was prepared for use in experiments as described for strain Pf-5.

Radicle assay. Cotton cultivar DES 119 was used in all experiments. Seeds were surface-disinfested by agitating at 100 rpm for 10 min in 30% H_2O_2, followed by four to five rinses in sterile water until bubbling ceased and drying in open petri dishes in a laminar flow hood. Prior attempts to achieve surface sterilization by treatment with 75-95% ethanol or with 1.5-5% NaClO were unsuccessful, and details are not presented here. Surface sterilization was confirmed by placing individual seeds into 3 ml TSB with shaking at 100 rpm for 12 h, followed by incubation for 96 h at 28 C.

Dried, surface-disinfested seeds were placed on water agar, five seeds per plate (plates 10 cm in diameter), and incubated for 48 h at 28 C. At this time, the mean radicle length was 1.5 cm. Prior attempts to germinate seeds in flasks containing sterile water resulted in inferior germination (details not presented here). Germinated seeds were individually transferred onto water agar plates. Radicles were inoculated with *R. solani* at a point midway from the seed to the radicle tip, using procedures described below (optimization of assays), and were incubated at 25 C.

Radicles were examined at 24-h intervals under a 10× dissecting microscope, and symptom development was ranked daily using the following rating scale: 0 = no symptoms, radicle completely white; 1 = radicle mainly white with some yellow discoloration; 2 = 50% of radicle surface yellow; 3 = some sunken yellow lesions showing; 4 = some brown lesions; 5 = some brown sunken lesions; 6 = over 50% of radicle with sunken lesions; and 7 = radicle completely brown and sunken.

Hypocotyl assay. Surface-disinfested cotton seeds were germinated in Promix (Premier Peat, Rivière-du-Loup, Québec, Canada). Promix was moistened and autoclaved twice before use. Hypocotyl sections 6 cm long were collected from 1- to 2-wk-old plants by cutting 3 cm above and 3 cm below the soil line. Lateral roots were excised from the hypocotyl, and hypocotyl sections were rinsed under running tap water to remove peat particles. Sections were surface-disinfested by shaking at 100 rpm for 10 min in 2% NaClO followed by eight rinses with sterile water, each with five times the original volume of the NaClO solution. Sterility checks were performed as described above. Hypocotyls were dried in open petri dishes in a laminar flow hood and were individually transferred to water agar plates.

Hypocotyls were inoculated with *R. solani* at a point 2 cm below the hypocotyl-radicle junction. Plates were incubated at 25 C and were rated at 24-h intervals for symptom development using the following rating scale: 0 = no symptoms, hypocotyl all white; 1 = hypocotyl mainly white with some tan; 2 = 50% of hypocotyl tan to yellow-brown; 3 = some browning at the hypocotyl-radicle junction; 4 = total browning of junction area; 5 = sunken lesions at junction; 6 = some browning below junction; and 7 = hypocotyl completely brown and sunken.

Optimization of assays. Test conditions for each assay were optimized for progressive development of symptoms over a 4- to 5-day period. More rapid symptom development was deemed inappropriate for screening biological control agents. Assay conditions that were tested included the form of *R. solani* inoculum, the growth medium for the pathogen, and the age of host tissue. All experiments described below consisted of five replicates, were separately conducted with the radicle and the hypocotyl assays, and were repeated one or two times with similar results. For all experiments included in this report, data were analyzed using SAS two-way analysis of variance. Treatment means were compared by calculating LSD at $P = 0.05$, and experimental variation was determined by calculating the mean standard error at the same probability.

Effect of inoculum form on symptom rating was tested using a noninoculated control and four inoculation treatments, including one half of a plug (4 mm in diameter) of *R. solani* grown on PDA and 10^0, 10^1, and 10^2 dilutions of a mycelial suspension. The mycelial suspension was prepared by mixing one 4-day-old PDA plate of *R. solani* in 50 ml of sterile water for 40 sec in a Waring blender on high speed. The mycelial suspensions were applied by placing two 40-µl drops onto hypocotyls or radicles.

R. solani inoculum was grown on seven media to determine effects on symptom development; these included PDA, water agar, and 10, 20, 30, 40, and 50% cotton seed exudate agar (CSEA). CSEA was prepared by amending water agar with the appropriate concentration of cotton seed exudates. Seed exudates were prepared by agitating individual surface-disinfested cotton seeds in 10 ml of sterile water in tubes for 3 days at room temperature. Tubes in which the water was cloudy were discarded. Water from the remaining tubes was passed through a 0.45 µm filter and stored at 4 C for up to 3 mo before use. *R. solani* was grown for 96 h at 25 C on each medium before inoculating hypocotyls or radicles with one half of a disk 4 mm in diameter. A noninoculated control treatment was included in each experiment.

The effect of tissue age on symptom development was determined using radicles and hypocotyls of various ages. For the radicle assay, five treatments were used, representing 0, 1, 2, 3, and 4 days after germination. Average radicle lengths for these treatments were 1.5, 3, 5, 6, and 7 cm, respectively. For the hypocotyl assay, seven treatments were used, representing 6, 7, 8, 9, 10, 11, and 12 days after planting, which corresponded to 2, 3, 4, 5, 6, 7, and 8 days after emergence, respectively. Inoculation for both assays was done using plugs of *R. solani* grown on 20% CSEA.

Experiments with known biological control agents. *Radicle assay.* All experiments with known biological control agents in the radicle assay involved five replications of each treatment and were repeated one or two times with similar results. In each experiment, symptoms were rated daily, using the scale described above, until 6 days after inoculation with *R. solani*.

The first experiment was designed to determine if the bioeontrol agents reduced *R. solani* symptom development. *E. coli* was used as a control bacterium lacking biological control activity. Bacterial suspensions were applied to radicles by spreading a 5-µl drop along the radicle length. Inoculated radicles were incubated for 12 h at 28 C before inoculation with *R. solani*, which was applied using plugs of mycelia grown on 20% CSEA (described above). All bacteria were evaluated with and without *R. solani*. A nontreated eontrol and a control of *R. solani* with no bacteria were included.

Because phytotoxicity was observed in the first experiment with Pf-5 (see results), the second experiment was designed to test the effect of bacterial concentration on phytotoxicity. Various concentrations of Pf-5 were applied to radicles, using the same technique as the first experiment, to give inoculation rates of log 1, 2, 3, 4, and 5 cfu per radicle. All rates were evaluated with and without *R. solani* inoculation, which was done with the same technique as the first experiment. A nontreated control and a control of *R. solani* with no bacteria were included.

The effect of timing of Pf-5 application on phytotoxicity was evaluated by dipping seeds into a bacterial suspension and observing the radicles after 48 h for discoloration. Treatments included nongerminated (freshly surfaee-disinfested) seeds, germinated seeds after 24 h on water agar (mean radiele length = 0.5 cm), and germinated seeds after 48 h on water agar (mean radicle length = 1.5 cm). Ten replications of each treatment were used. Based on the results, an experiment was designed to test the effect of timing of Pf-5 application on biological control activity, as

measured by the rate of symptom development. Nongerminated seeds treated with Pf-5 and seeds that had been on water agar for 24 h were inoculated with *R. solani*; a control of *R. solani* without bacteria was included.

The effect of timing of GBO-3 application on symptom development also was evaluated in a separate experiment. The same seed ages were used as with Pf-5. A nontreated control and a control of *R. solani* with no bacteria were included.

Experiments with known biological control agents. *Hypocotyl assay.* The experiments with known biological control agents in the hypocotyl assay used five replications of each treatment and were repeated once or twice with similar results. In each experiment, symptoms were rated daily, using the scale described above, until 6 days after inoculation with *R. solani.*

An initial experiment was performed to determine if strains Pf-5 and GBO-3 reduced *R. solani* symptom development. Hypocotyls were inoculated by pipeting 15 μl of a bacterial suspension along the length of the hypocotyl. After bacterial application, hypocotyls were incubated for 12 h at 28 C before inoculation with *R. solani. E. coli* was added as a control bacterium lacking biological control activity. All bacteria were tested with and without *R. solani*, which was applied using plugs of mycelia grown on 20% CSEA (described above). A nontreated control and a control of *R. solani* with no bacteria were included.

The effect of timing of bacterial application on biological control activity was determined. Treatments included Pf-5 or GBO-3 with *R. solani* inoculated immediately after, 24 h after, and 48 h after application of bacteria. Control treatments included Pf-5 and GBO-3 without *R. solani* and *R. solani* without bacteria.

Bacterial population dynamics and relation to biological control. Experiments were designed to determine the relation of bacterial inoculum density to biological control and to assess final baeterial populations on hypocotyls and radicles. Serial dilutions of rifampicin-resistant mutants of strains Pf-5 and GBO-3 were prepared and used to treat hypocotyls and radicles in separate experiments. The applied populations in the hypocotyl assay were log 0.58, 1.58, 3.58, 5.58, and 6.58 cfu per hypocotyl for PF-5 and log 0.52, 2.52, 4.52, 6.52, and 7.52 cfu per hypocotyl for GBO-3. In the radicle assay, applied populations were log 1.9, 3.9, 5.9, and 6.9 cfu per radiele with Pf-5 and log 1.96, 3.96, 5.96, 7.96, and 8.96 cfu per radicle for GBO-3. A nontreated control and a control of *R. solani* with no bacteria were included in each experiment. All treatments were replicated five times. Symptoms were rated as in previous experiments, and the final mean cfu per radicle or cfu per hypocotyl was determined by plating serial dilutions on TSA + 100 mg/L rifampicin using a spiral plater (Spiral Systems, Bethesda, MD). After incubation at 28 C for 24 (Pf-5) or 48 h (GBO-3), bacteria were enumerated with a laser colony counter and bacterial enumeration software (Spiral Systems).

RESULTS

Optimization of assays. The form of *R. solani* inoculum had a significant effect on symptom development in both the radicle and hypocotyl assays (Fig. 1). In the radicle assay (Fig. 1A), all forms of inoculum resulted in similar symptom development for the first 2 days after inoculation. Symptom ratings for the undiluted and diluted mycelial suspensions were similar from day 3 to day 6. Inoculation with PDA plugs resulted in total necrosis of hypocotyls (rating = 7) by day 5. In the hypocotyl assay (Fig. 1B), inoculation with the undiluted mycelia suspension resulted in rapid development of symptoms with an average rating of 4.0 24 h after inoculation and 5.7 on day 3. With both dilutions of the mycelia suspension, symptom ratings were significantly less, but the average ratings 6 days after inoculation were similar to the noninoculated control. Inoculation with PDA plugs resulted in symptom development intermediate between the undiluted and diluted mycelial suspensions. Based on these results, radicles and hypocotyls in all subsequent experiments were inoculated by the plug-treatment technique.

Effects on symptom development also varied with the media used to grow *R. solani* (Fig. 2). In the radicle assay (Fig. 2A), symptom ratings were highest with inoculum from PDA and 10% CSEA, intermediate with 20% CSEA, and lowest with 30, 40, and 50% CSEA. In the hypocotyl assay (Fig. 2B), all media except water agar resulted in similar symptom development over the course of the assay. With water agar, symptom ratings were less than all other treatments from day 1 to day 3. Based on the results, 20% CSEA was selected for use in all subsequent experiments in both the radicle and hypocotyl assays.

The age of cotton tissue inoculated with *R. solani* had little

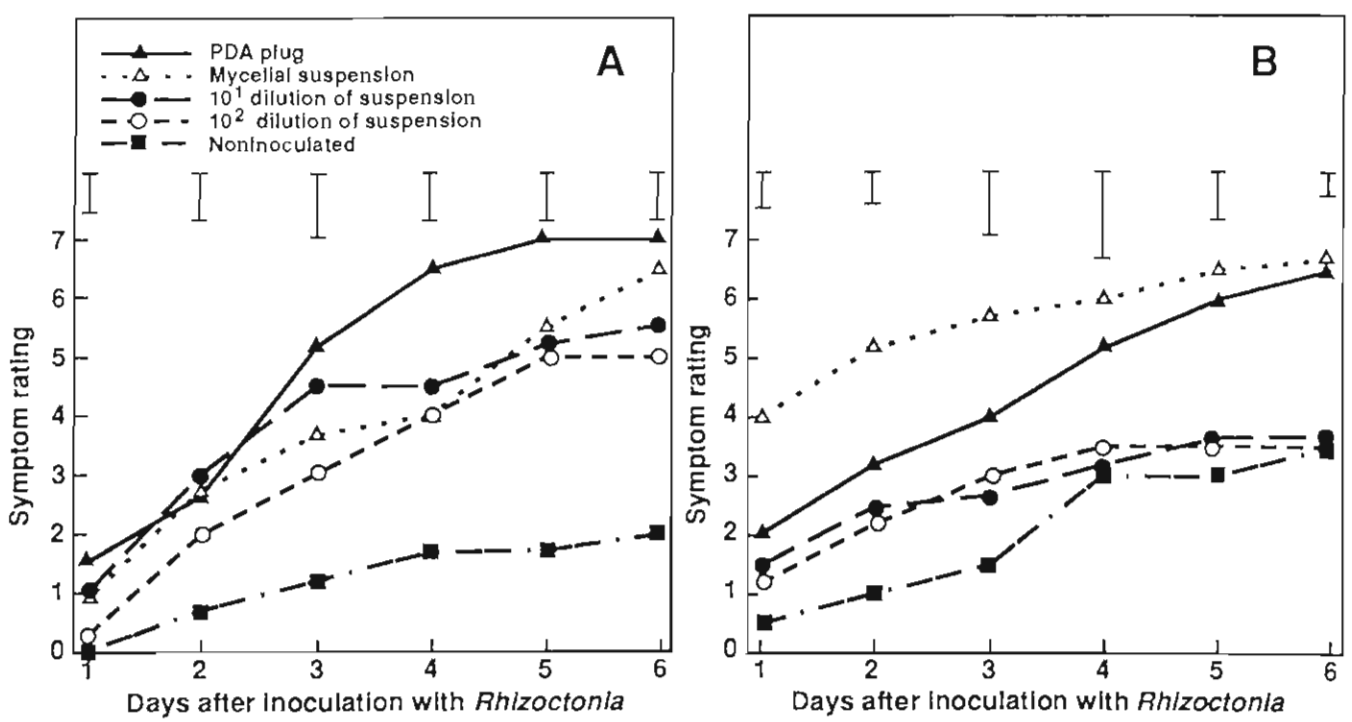

Fig. 1. Effect of the form of *Rhizoctonia solani* inoculum on damping-off symptom ratings. Symptoms were rated with a scale of 0–7, where 0 = no symptoms and 7 = total necrosis. Bars = mean standard error. A, radicle assay. B, hypocotyl assay.

effect on symptom development (Fig. 3). In the radicle assay (Fig. 3A), symptom ratings were highest for the first 3 days after inoculation with seeds 1 day after germination. Final symptom ratings were similar for all ages, with a range of average ratings 6 days after *R. solani* inoculation of 4.7–5.3. The tested ages of hypocotyls had no pronounced effect on average final symptom rating (Fig. 3B).

Experiments with known biological control agents. *Radicle assay.* In the first radicle assay with known biological control agents (Fig. 4), strain GBO-3 demonstrated biological control activity as evidenced by a reduction in the final average symptom rating compared to the *R. solani* control. Symptom ratings at 6 days after *R. solani* inoculation were 2.8 and 5.0 for GBO-3 and the *R. solani* control, respectively. Strain Pf-5 induced phytotoxicity when applied alone and with *R. solani*. Symptom ratings at 6 days after pathogen inoculation were 5.8 and 5.0 for Pf-5 and the pathogen control, respectively. *E. coli* treatment resulted in neither biological control nor phytotoxicity.

The second experiment evaluated the effect of Pf-5 concentration on the observed phytotoxicity by using bacterial concentrations of log 1, 2, 3, 4, and 5 cfu per radicle. Phytotoxicity was noted with all Pf-5 concentrations. As in the first experiment, both treatments of Pf-5 alone and Pf-5 with *R. solani* inoculation resulted in phytotoxicity. Radicles treated with Pf-5 alone showed yellow-brown lesions 36 h after bacterial inoculation with concentrations of log 3–5 cfu per radicle and 60 h after inoculation with concentrations of log 1–2 cfu per radicle.

Initial evaluation of the effect of timing of Pf-5 inoculation on phytotoxicity revealed discoloration of radicles 48 h after application to seeds in different stages of germination. Based on

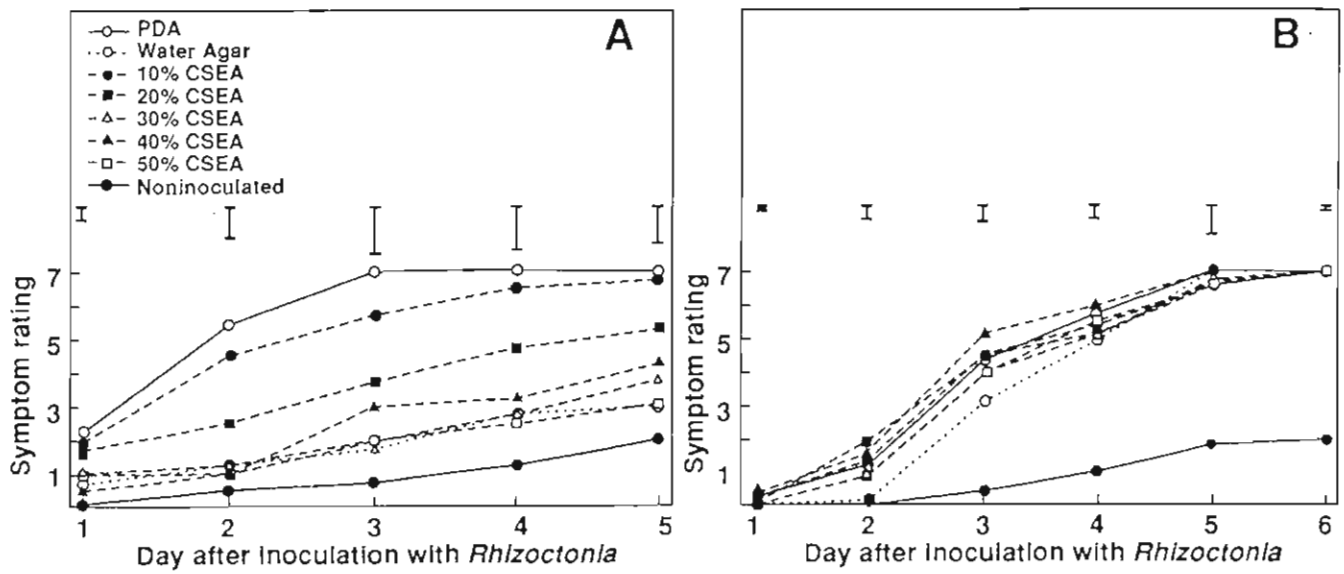

Fig. 2. Effect of *Rhizoctonia solani* growth media on damping-off symptom ratings. All inoculations were with mycelial plugs using the indicated media. CSEA = cotton seed exudate agar. Bars = mean standard error. **A**, radicle assay. **B**, hypocotyl assay.

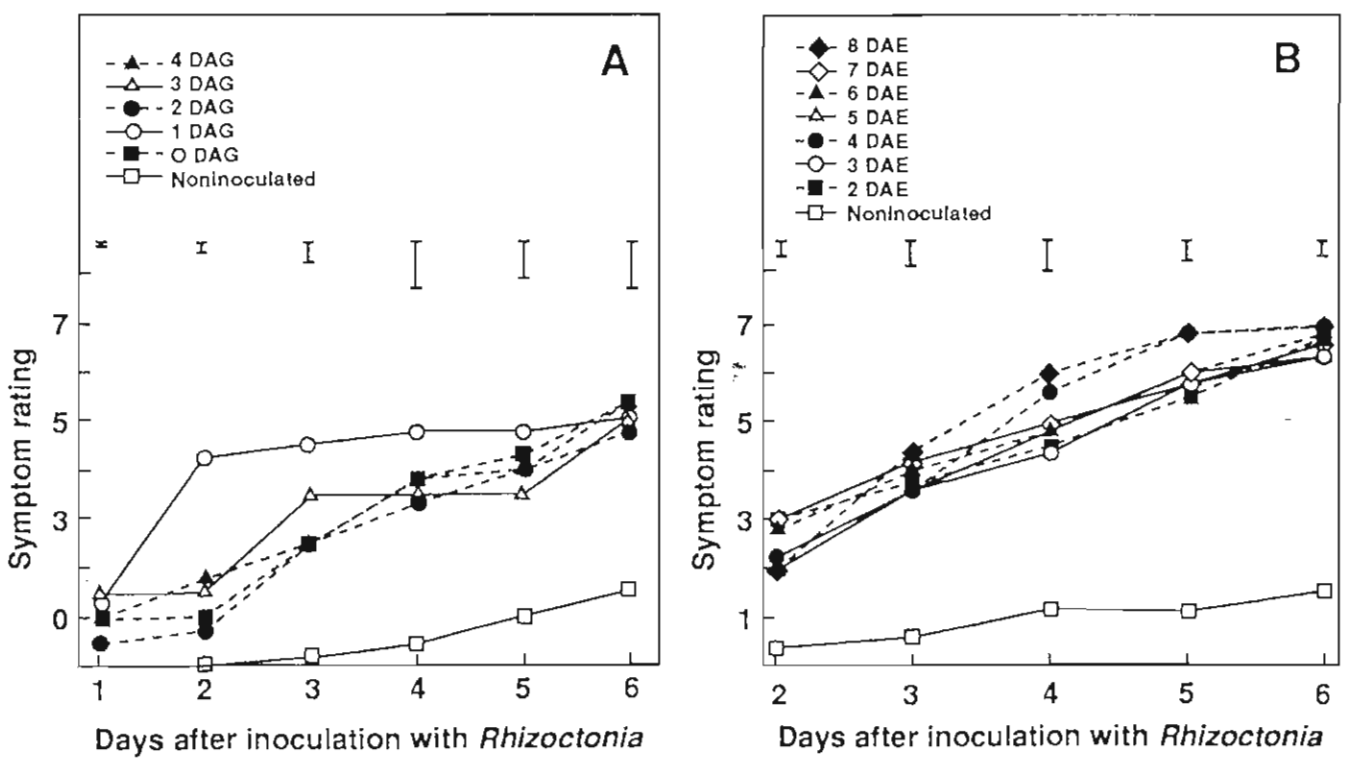

Fig. 3. Effect of cotton tissue age on damping-off symptom ratings. DAG = days after germination. Bars = mean standard error. DAE = days after emergence. **A**, radicle assay. **B**, hypocotyl assay.

these results, nongerminated seeds and seeds germinated for 24 h treated with Pf-5 were evaluated for biological control activity by inoculating with *R. solani* (Fig. 5). Pf-5 on nongerminated seeds demonstrated biological control by reducing symptom development compared to the *R. solani* control. In contrast, Pf-5 on seeds germinated for 24 h showed no biological control and had higher symptom ratings than the control 1 and 2 days after inoculation of *R. solani*.

The same stages of seed germination that were evaluated with Pf-5 were used to test biological control with GBO-3 (Fig. 6). GBO-3 reduced symptom development on nongerminated seeds and seeds germinated for 24 h to a level similar to the control without *R. solani*. The reduction in symptom development was less marked for seeds germinated for 48 h.

Experiments with known biological control agents. *Hypocotyl assay.* In the initial evaluation of biological control activity in the hypocotyl assay (Fig. 7), bacteria were applied 12 h before inoculation with *R. solani*. Strain Pf-5 demonstrated biological control, whereas strain GBO-3 and *E. coli* did not. Mean symptom ratings 6 days after inoculation with *R. solani* were 3.0, 6.8, 7.0, 7.0, and 1.0 for Pf-5, GBO-3, *E. coli*, the *R. solani* control, and the nontreated control, respectively. No phytotoxicity was evident by treatments with the bacteria alone.

In tests for effect of timing of bacterial application on biological control activity (Fig. 8), strains Pf-5 and GBO-3 both resulted in marked reduction of symptom development following inoculation of hypocotyls with *R. solani* immediately after drying the applied bacterial suspensions. Mean symptom ratings 6 days after inoculation with *R. solani* were 1.0, 1.6, and 6.0 for Pf-5, GBO-3, and the *R. solani* control, respectively. When *R. solani* was applied

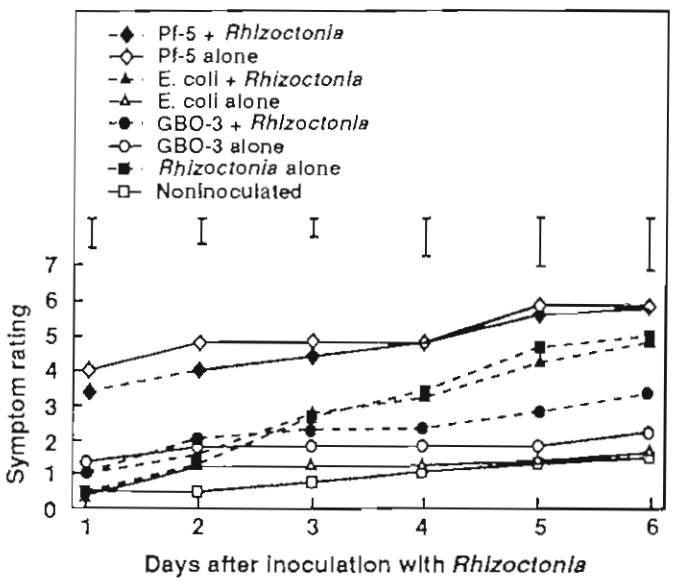

Fig. 4. Evaluation of biological control bacterial strains *Pseudomonas fluorescens* Pf-5 and *Bacillus subtilis* GBO-3 in the radicle assay. Bacteria were applied 12 h before inoculation with *Rhizoctonia solani*. *Escherichia coli* served as a control bacterium lacking biological control activity. Bars = mean standard error.

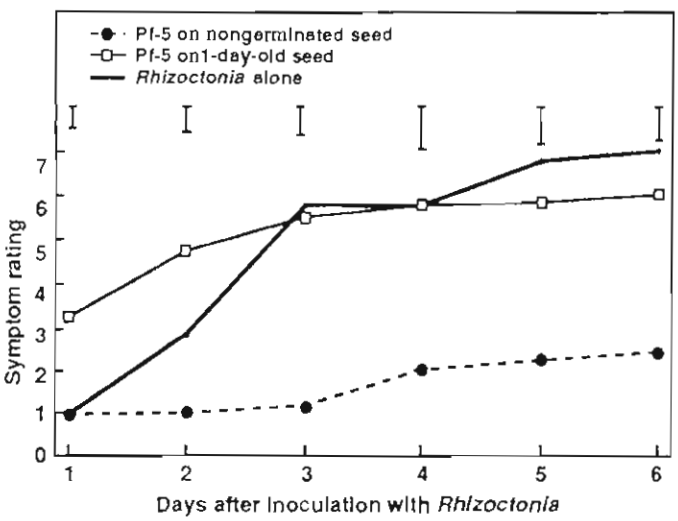

Fig. 5. Effect of timing of *Pseudomonas fluorescens* Pf-5 application on development of symptoms in the radicle assay. Bacteria were applied to nongerminated seed and seed 24 h after germination before inoculating with *Rhizoctonia solani*. Bars = mean standard error.

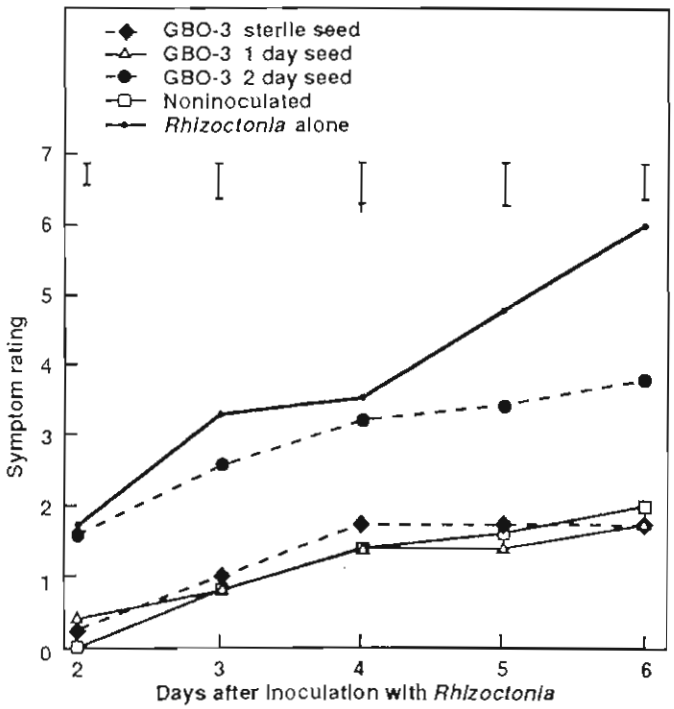

Fig. 6. Effect of timing of *Bacillus subtilis* GBO-3 application on development of symptoms in the radicle assay. Bacteria were applied to nongerminated seed and seed germinated 24 and 48 h before inoculation with *Rhizoctonia solani*. Bars = mean standard error.

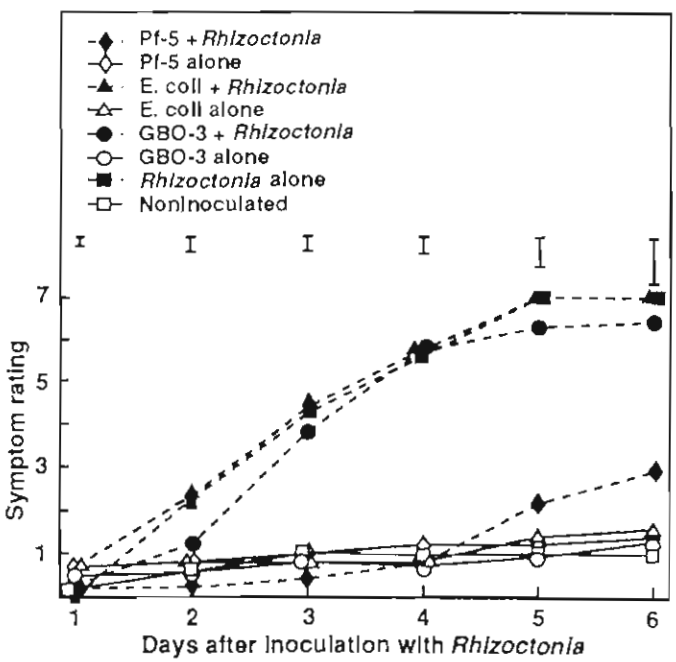

Fig. 7. Evaluation of biological control bacterial strains *Pseudomonas fluorescens* Pf-5 and *Bacillus subtilis* GBO-3 in the hypocotyl assay. Bacteria were applied 12 h before inoculation with *Rhizoctonia solani*. *Escherichia coli* served as a control bacterium lacking biological control activity. Bars = mean standard error.

24 and 48 h after the PF-5 and GBO-3 treatments, higher ratings of symptom development with time and higher final ratings 6 days after pathogen inoculation resulted. Inoculation of bacteria but no *R. solani* resulted in final 6-day ratings of 1.0 and 1.6 for Pf-5 and GBO-3, respectively.

Bacterial population dynamics and relation to biological control. Strain Pf-5 multiplied on radicles during the 6-day assay over the complete range of tested inoculum densities (Fig. 9A). Inoculation with log 1.9 cfu per radicle resulted in a final population of log 6.7 cfu per radicle, whereas inoculation with log 6.9 cfu per radicle resulted in a final population of log 8.0 cfu per radicle. In contrast, final populations of strain GBO-3 were higher than the inoculated population using inoculum densities of log 2, 4, and 6 cfu per radicle but were lower than the inoculated populations using inoculum densities of log 8 and 9 cfu per radicle. Inoculation with log 2 cfu per radicle resulted in a final population of log 5.1 cfu per radicle, whereas inoculation with log 9 cfu per radicle resulted in a final population of log 7.5 cfu per radicle.

Bacterial inoculum density on radicles also affected the biological control response (Fig. 9A). With strain Pf-5, symptom ratings 6 days after inoculation with *R. solani* decreased steadily with initial populations of log 4–7 cfu per radicle, whereas, with GBO-3, symptom ratings did not change from log 6 to 9 cfu per radicle. Protection of *R. solani*-inoculated radicles, resulting in reduced necrosis after seed treatment with Pf-5 and GBO-3, is shown in Figure 10.

Similar results occurred in studies of bacterial population dynamics in the hypocotyl assay (Fig. 9B). Strain Pf-5 was recovered at higher populations than inoculated over the complete range of inoculum densities. Inoculation with log 0.6 cfu per hypocotyl resulted in a final population of log 6.3 cfu per hypocotyl, whereas inoculation with log 6.6 cfu per hypocotyl resulted in a final population of log 9.6 cfu per hypocotyl. Inoculum density of GBO-3 had a similar effect on final populations. Inoculation with log 0.5 cfu per hypocotyl resulted in a final population of log 2.0 cfu per hypocotyl, whereas inoculation with log 7.5 cfu per hypocotyl resulted in a final population of log 4.0 cfu per hypocotyl.

Inoculum density had a strong effect on biological control activity with strain Pf-5 but much less effect with strain GBO-3 (Fig. 9B). Symptom ratings declined steadily with PF-5 inoculum densities of log 0.5, 2.5, 4.5, and 6.5 cfu per hypocotyl. With strain GBO-3, symptom ratings declined slightly with inoculum densities from log 0.5 to 2.5 cfu per hypocotyl but showed no overall change with inoculum densities from log 4.5 to 7.5 cfu per hypocotyl.

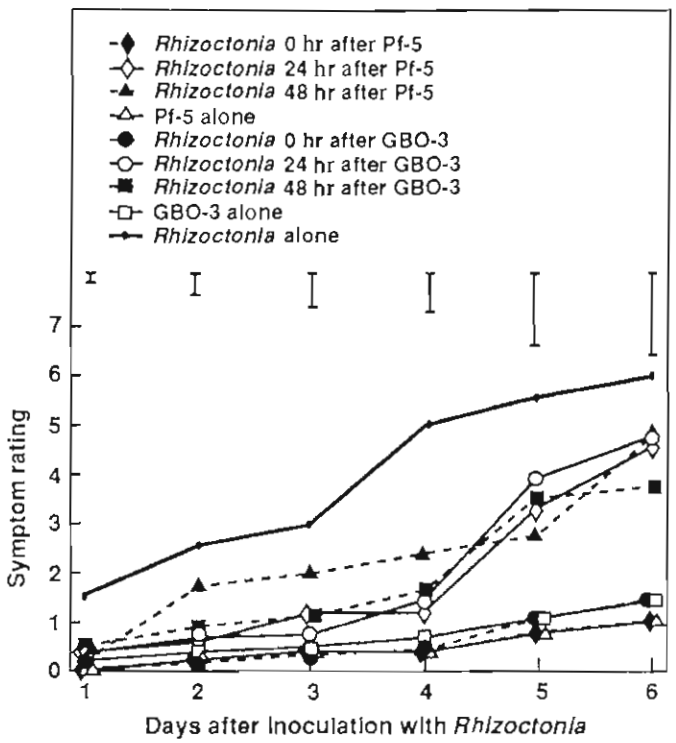

Fig. 8. Effect of timing of bacterial application on development of symptoms in the hypocotyl assay. *Rhizoctonia solani* inoculum was applied immediately after, 24 h after, and 48 h after application of bacteria. Bars = mean standard error.

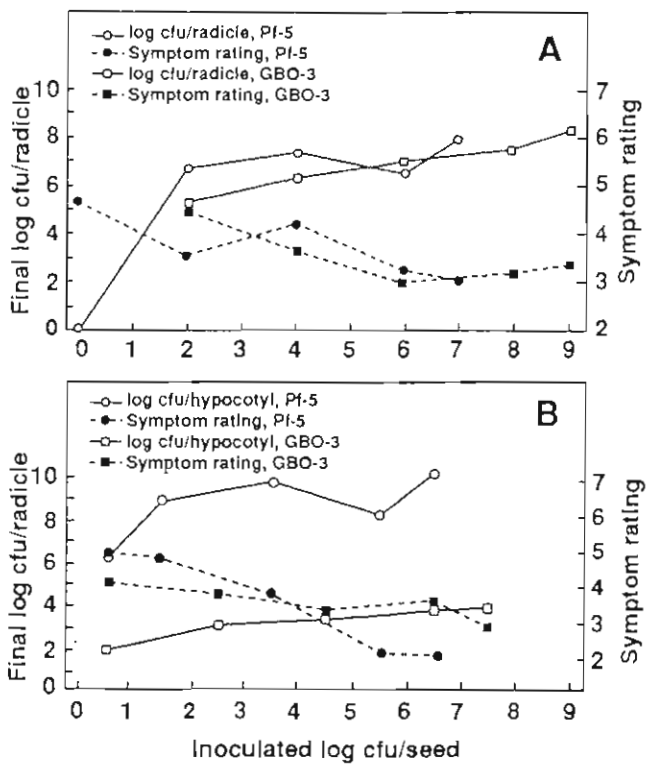

Fig. 9. Population dynamics of *Pseudomonas fluorescens* Pf-5 and *Bacillus subtilis* GBO-3 on radicles and hypocotyls–the effect of bacterial inoculum density on final bacterial populations and on symptom development. A, radicle assay. B, hypocotyl assay.

DISCUSSION

Two in vivo assays were developed for prescreening candidate biological control agents of *R. solani* on cotton. A radicle assay was designed as a prescreen for preemergence damping-off and a hypocotyl assay for postemergence damping-off. Recording mean symptom ratings at 24-h intervals for 6 days after inoculation with *R. solani* allowed comparisons of treatments based on symptom ratings at each observation time. Two previously reported bacterial biological control agents of *R. solani* demonstrated biological control, as measured by reduction in final symptom ratings, whereas *E. coli* had no effect, thereby validating the potential

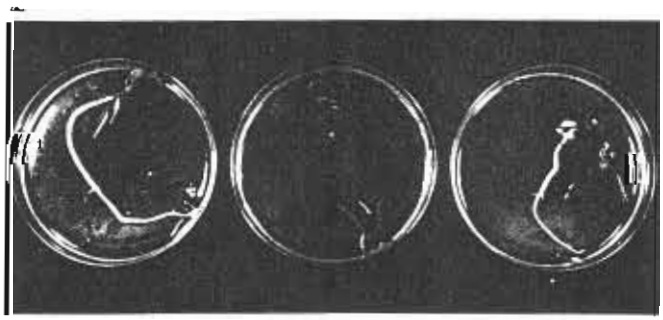

Fig. 10. Effect of biological control agents on development of symptoms caused by *Rhizoctonia solani* on cotton radicles. Center, *R. solani* control with no bacteria. Right, bacterial strain *Bacillus subtilis* GBO-3 with *Rhizoctonia solani*. Left, strain *Pseudomonas fluorescens* Pf-5 with *R. solani*. Photograph was taken 6 days after inoculation with *R. solani*.

usefulncss of the assays in selection strategics for biological control agents.

Experiments with different assay parameters (Figs. 1–3) optimized the assay conditions such that symptoms developed progressively over 6 days, so that biological agents could be assessed over time for control activity. Treatment with mycelial plugs of *R. solani* on agar was sclected as the inoculation form since this treatment resulted in a lower symptom ratings at early observation times compared to inoculation with undiluted mycelial suspensions (Fig. 1).

The media used to grow *R. solani* inoculum had a pronounced effect on symptom development in the radicle assay but not in the hypocotyl assay (Fig. 2). This confirms previous reports that the nutritional status of inoculum affects *R. solani* virulence (26). Growing inoculum on agar containing cotton seed exudates (CSEA) resulted in more rapid symptom development compared to growth on water agar in the radicle assay (Fig. 2A). This agrees with previous studies that suggested that exudates favor disease development. Nour El Dein and Sharkas (15) found that disease severity in tomato cultivars is correlated with stimulation of fungal growth by seed exudates. Schroth and Cook (21) reported that the incidence of preemergence damping-off is influenced by seed exudates and concluded that, "although there may be some differences in quality of exudates, the quantity appeared the important factor." In the radiele assay, quality of exudatcs appears to be a contributory factor since amendment of water agar with 40–50% cotton seed exudates repeatedly resulted in less disease (lower symptom ratings) than 10–20% exudates. The age of seedlings had little effeet on symptom development (Fig. 3), indicating that the radicle and hypocotyl assays can be eonducted for several days using the same plant material, thereby simplifying the logistics of the assays.

Phytotoxicity was evident with *P. fluorescens* strain Pf-5 in initial experiments testing the effect of known biologieal control agents in the optimized radicle assay (Fig. 4). Subsequent experiments with seedling age indicated that the phytotoxicity occurred only when radicles of pregerminated seeds were inoculated with Pf-5. Inoculation of nongerminated seeds resulted in no evident phytotoxicity and provided significant biologieal control (reduction in the rate of symptom development and final symptom rating compared to the *R. solani* control) (Fig. 5). This treatment represents the stage when seeds would be inoculated under normal agronomic conditions. With *B. subtilis* strain GBO-3, no phytotoxicity was observed at any tested seedling ages, although biological control activity was maximum with inoculation of nongerminated seeds (Fig. 6).

In the initial evaluation of Pf-5 and GBO-3 in the hypocotyl assay (Fig. 7), in which bacteria were applied to hypocotyls 12 h before inoculation with *R. solani*, biological control activity resulted from treatment with strain Pf-5 but not with GBO-3. In a subsequent experiment evaluating the effect of timing of bacterial applieation (Fig. 8), biological control resulted with both bacterial strains when *R. solani* was applied immediately after drying the bacterial inoculum. Based on the experiments conducted in this study, it is unknown why biological control was optimal when bacterial inoculated hypocotyls were challenged immediately with *R. solani*. Three explanations are considered: bacterial populations declined at 24 and 48 h after application; the active bacterial compound aceounting for biologieal control was produeed in lower amounts with time; hypocotyl exudates change with time, possibly by bacterial metabolism of specific exudate constituents resulting in an altered ratio of compounds in exudates, which may result in enhanced virulence of *R. solani*.

Reductions in bacterial populations on hypoeotyls over time does not appear to be a factor based on results from the population dynamics study (Fig. 9). Both bacterial biological control agents were detected on hypocotyls and radicles 6 days after application. The population increase during the 6-day assay depended on both the strain and the baeterial inoculum density. Strain GBO-3 reached maximum populations of log 5–7 and log 2–4 on radicles and hypocotyls, respectively. When inoculated at lower populations, GBO-3 increased to these maximum levels, whereas it decreased when inoculated at higher population densities. In contrast, the population of Pf-5 increased over all tested inoculum densities in both the radicle and the hypocotyl assays. The results do not preclude the possibility that the initial populations decreased after application and then recovered to the final populations.

The inoculum density of bacteria also influenced the magnitude of biological control (Fig. 9). With strain Pf-5, optimum biological control occurred with the highest inoculum densities, whereas with strain GBO-3, equivalent symptom ratings occurred over a range of inoculum densities. When the symptom ratings are compared to the final population on radicles or hypocotyls at various inoculum densities, it is apparent that achieving relatively high host colonization does not necessarily translate to efficacy. For example, strain Pf-5 in the hypocotyl assay (Fig. 9B) had final populations above log 8 cfu per hypocotyl with initial inoculum densities of log 1.5, 2.5, 3.5, 5.5, and 6.5 cfu per hypocotyl; however, the disease ratings declined progressively with increasing inoculum densities from log 1.5–5.5 cfu per hypocotyl. A similar trend was reported by Lifshitz et al (11) in an assay with plant growth-promoting rhizobacteria. *P. putida* strain GR 12-2 colonized roots at populations of log 3–3.5 cfu/cm of root with inoculum densities of log 1.9 cfu/ml or above, but efficacy (measured by promotion of root elongation) was optimal at higher inoculum densities.

The use of in vivo assays similar to those reported here may be more advantageous than prescreens involving antibiosis in vitro, as in vitro activity does not allow for interactions with the host tissues. As pointed out by Cook and Baker, "the host plant is a participant in virtually any biological control aimed at suppression of the disease-producing activities of the pathogen" (2). These authors also state that "antagonism on the plant surface involves mainly competition and antibiosis but also may involve mycoparasitism and predation." In addition, induced resistance mechanisms would only be operable when the host plant is used in selection assays. Therefore, in order to assess all possible means of antagonism by potential biological control agents, it is necessary to include the host in the assessment.

Including the host in evaluations of biological control potential by microorganisms is especially important with *R. solani*. Plant materials play a key role in determining fungal virulence and disease severity with *Rhizoctonia* (3). *Rhizoctonia* isolates that cause damping-off commonly penetrate the host from infection cushions that are morphologically distinct from hyphae and that require intensive metabolic aetivity during formation (3). Exudates from seeds (12) and stems (4) were found to diffuse through membranes and synthetic films and induce cushion formation by *R. solani* hyphae. Hence, one potential scenario for biological control, which would not relate to antibiosis in vitro, would be a reduction or alteration in the plant exudate constituents by the biocontrol agent resulting in the inability of *R. solani* to form infeetion cushions.

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