Comparison of *Pseudomonas* Species and Application Techniques for Biocontrol of Rhizoctonia Stem Rot of Poinsettia

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ABSTRACT

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Pseudomonas cepacia (strain 5.5B) was compared with other strains of *P. cepacia, P. fluorescens, P. chlororaphis,* and *P. aureofaciens* for biocontrol of Rhizoctonia stem rot of poinsettia caused by *Rhizoctonia solani* in polyfoam rooting cubes. Over a 2-wk period, complete control (0% infection) of stem rot was achieved with *P. cepacia,* strain 5.5B. With other strains of *P. cepacia,* infection ranged from 0 to 93%. Infection ranged from 63 to 97% with all other strains of *Pseudomonas* spp. Different application methods for delivering strain 5.5B for stem rot control were tested. Rooting cubes soaked with a suspension of strain 5.5B or cubes soaked with a bacterial suspension followed by a bacterial spray over-the-top of cuttings (at day 0) were the most effective application methods. Cubes soaked with water followed by a bacterial spray over-the-top of the cuttings in cubes controlled ($P = 0.05$) stem rot compared with the control. Three spray applications of *P. cepacia* strain 5.5B to cuttings during a 2-wk period were more effective than either one or two bacterial sprays in control of Rhizoctonia stem rot. Spraying poinsettia stock plants with a suspension of strain 5.5B prior to taking cuttings, or dipping cutting stems in a bacterial suspension prior to placing cuttings in cubes, provided no stem rot control.

Strains of *Pseudomonas* spp. have been studied extensively as biocontrol agents of plant diseases (6,16,20,36). Some strains have been particularly effective for controlling several soilborne pathogens (5,17,22,24,35). Because these bacteria possess many desirable attributes, their potential for use in disease control strategies is substantial (6).

A primary constraint in using biocontrol agents is the absence of a suitable delivery or application system. Many potential biocontrol agents are deemed unsuitable because the method of delivery is impractical, laborious, or expensive (2,15). An effective antagonist should be somewhat adaptable to existing systems (i.e., spray programs) in terms of equipment use, frequency of application, and cost effectiveness (36).

The more controlled environmental conditions in greenhouses coupled with increasing restrictions on the use of chemical pesticides provide favorable circumstances for using biocontrol strategies in greenhouse ornamental production (3,11,23,29,32,33). Poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch) is a principal crop in the greenhouse industry with production continuing to expand in the U.S. In 1992, the wholesale value of poinsettias reached almost 200 million dollars (34). Poinsettias are affected by many diseases, including Rhizoctonia stem rot, caused by *Rhizoctonia solani* Kuhn (teleomorph = *Thanatephorus cucumeris* (Frank) Donk) (19,30). Stem rot is most prevalent during the rooting stage of production.

Many poinsettia cuttings are rooted in artificial (polyfoam), soilless rooting cubes that provide for efficient use of space and uniform root development (13). The conditions under which cuttings are rooted, however, and the environment of the cube provide a conducive infection court for *R. solani.* Although control involves sanitation and fungicides, stem rot continues to occur at endemic and occasionally epidemic levels (1,19,25,27,30).

Recently, a strain of *Pseudomonas cepacia* (strain 5.5B, ATTC 55344) was tested and characterized as an effective biocontrol agent of Rhizoctonia stem rot of poinsettia in polyfoam rooting cubes (8,9). The objectives of this research were to compare strains of *Pseudomonas* spp. to strain 5.5B of *P. cepacia* for their ability to inhibit *R. solani* in vitro and control Rhizoctonia stem rot in polyfoam rooting cubes, and to develop a practical delivery system for application of this strain to polyfoam rooting cubes for control of *R. solani.*

MATERIALS AND METHODS

Stock plants. Poinsettia stock plants (Gutbier V-14 Glory [red]) were maintained in 220 Metro mix (W. R. Grace Co., Cambridge, MA) contained in 6-L plastic pots or 22-cm-diameter clay pots on greenhouse benches. Stock plants were fertilized weekly by applying soil drenches of commercial potassium nitrate (100 ppm N), calcium nitrate (200 ppm N), and 20-10-20 fertilizer (N-P-K, 300 ppm N). Magnesium sulfate (2.4 g/L) was applied as a drench each month. About every 3 mo, a foliar spray of 1% molybdenum was applied. A one time application of commercial soluble trace elements (S.T.E.M., 0.6 g/L) was applied as a drench. Stock plants were occasionally pruned to facilitate new growth.

Sources of *Pseudomonas* strains. Strains of *Pseudomonas cepacia* were obtained from K. E. Conway, Oklahoma State University, Stillwater, OK (strain OK2); J. W. Kloepper, Auburn University, Auburn, AL (strain JM69, strain JM388); R. D. Lumsden, USDA, ARS, Beltsville, MD (strain PCPS1); and Stine Microbial Products, Madison, WI (strain A, strain B). Strain PF-5 of *Pseudomonas fluorescens* was obtained from C. R. Howell, USDA, ARS, College Station, TX. Strains of *Pseudomonas fluorescens* (strain 2-79), *Pseudomonas chlororaphis* (ATCC 9446), and *Pseudomonas aureofaciens* (ATCC 13985) were obtained from L. S. Thomashow, Washington State University, Pullman, WA. All strains were grown on fresh potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) or King's medium B (KB) (12). Cells from these cultures were stored in sterile, deionized water at 4 C for later use.

In vitro inhibition of *R. solani* by *Pseudomonas* strains. Cells of *Pseudomonas* strains were retrieved from water-stored cultures and a single drop (~0.1 ml) of suspension was placed at the inside apex of each section of a compartmentalized (four compartments) petri dish (Fisher Scientific, Pittsburgh, PA) containing PDA. Plates were incubated on a laboratory bench at ambient temperature. After an incubation period of 2 wk, a 5-mm mycelial plug taken from an actively growing colony of *R. solani* (isolate RS-3 from poinsettia, AG 4, NRRL 22805) was placed opposite the bacterial colony at the outside edge of each section of the petri dish. Colony growth (measured from the outside edge of the petri dish to the leading edge of the colony) of *R. solani* was measured daily for 3 days. Each section of each plate was considered a replication.

Inoculum of *R. solani.* For all greenhouse experiments, rice was colonized

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with isolate RS-3 of *R. solani* by seeding twice autoclaved rice contained in 250-ml flasks (25 g of rice per 18 ml of water) with two mycelial plugs taken from the margin of an actively growing colony of *R. solani*. Flasks were incubated on a laboratory bench for 7–11 days before use.

Comparison of *Pseudomonas* strains for stem rot control. All strains were retrieved from water-stored cultures and grown on fresh KB. After 8–14 days, cells from each strain were used to seed 500 ml of nutrient broth (Difco Laboratories, Detroit, MI) in 1-L flasks. Flasks were placed on a rotary shaker (Barnstead/Thermolyne, Dubuque, IA) at 175 rpm for 3 days at ambient temperature. Bacterial cells were concentrated by centrifugation (~2,500 *g* for 15 min) and resuspended in deionized water. Suspensions were diluted and calibrated with a spectrophotometer (Spectronic 20D, Milton Roy, Rochester, NY) at 530 nm. Concentration for strains ranged from about log 8.7 to 9.2 cfu per milliliter.

Dry rooting cube strips (Oasis Rootcubes, Smithers-Oasis, Kent, OH) were soaked in plastic trays with the bacterial suspensions. Individual cubes (25 × 51 mm long × 37 mm high [47 cm^3]) completely absorbed 40 ml of liquid suspension in a few seconds. After soaking, cube strips (five cubes per strip = a replicate) were fitted with a styrofoam sleeve on the sides and bottom, secured with two rubber bands, and placed on greenhouse benches. Rooting cubes for infested and noninfested controls were soaked with deionized water. A rice grain colonized by *R. solani* was placed on top between cubes where cubes join (six grains per strip). Poinsettia cuttings (5–8 cm in length) taken from stock plants were placed in cube holes about 2 cm from rice grain and immediately misted. A misting regime of 1 min of mist per hour, 14 times a day was used.

Comparison of application methods of strain 5.5B of *P. cepacia* for stem rot control. Methods for growth and preparation of strain 5.5B were consistent with those in the previous section. Several methods of applying strain 5.5B to rooting cubes were compared. Poinsettia cuttings were placed in rooting cubes (five-cube strips) that had been soaked with 40 ml of deionized water per cube and infested with *R. solani* as previously described. The cuttings were then sprayed uniformly over-the-top with a log 9 cfu per milliliter suspension of strain 5.5B at a volume of 20–25 ml (per five-cube strip) from a 7.5-L hand-pressurized sprayer. In some treatments, the strips of cuttings were sprayed again with a suspension of strain 5.5B 7 days after initial soaking, or 5 and 10 days after initial soaking for a total of three applications. Controls included rooting cubes soaked with a suspension of 5.5B or deionized water.

In other experiments, stems of poinsettia cuttings were quick-dipped in a suspension of strain 5.5B at log 9 cfu per milliliter, then placed in rooting cubes soaked with deionized water. A variation of this treatment was spraying poinsettia stock plants with a suspension of strain 5.5B (until run-off) immediately prior to taking cuttings and placing in rooting cubes. In other treatments, the bottom half of cube strips were soaked with 20 ml of deionized water per cube followed by spraying the top half of cube strip with a suspension of strain 5.5B (20 ml per cube). A combination of soaking rooting cubes with a suspension of strain 5.5B followed by an immediate over-the-top spray application of strain 5.5B once cuttings and rice grains were placed was also used. Controls included rooting cubes soaked with deionized water.

Experimental design and statistical analysis. A randomized block design was used for all greenhouse experiments. Three replicates with five cubes per replicate were used for each treatment. For all greenhouse experiments, percent infection and mortality (based on number of diseased or dead cuttings) and disease and root development of cuttings were assessed at the end of 2 wk. Each series of experiments was conducted twice. Cuttings were rated for disease severity based on the following scale: 1 = no disease; 2 = lesions covering ≤ 25% of stem; 3 = lesions covering 26 ≤ 50% of stem; 4 = lesions covering 51 ≤ 75% of stem; and, 5 = stem completely girdled or collapsed. Root development was evaluated as follows: 0 = cutting collapsed; 1 = no infection of cutting stem or stem infected but not collapsed with no root initials present; 1.5 = beginning of callus formation; 2 = distinct root initials; 2.5 = callus completely encircling the stem; 3 = callus tissue plus beginning of lateral roots; and, 3.5 = callus plus distinct lateral roots. Experimental data were pooled based on homogeneity of variance. All data were analyzed with PC SAS (SAS Institute, Cary, NC) with PROC GLM. Means were separated by the Waller-Duncan *k*-ratio test.

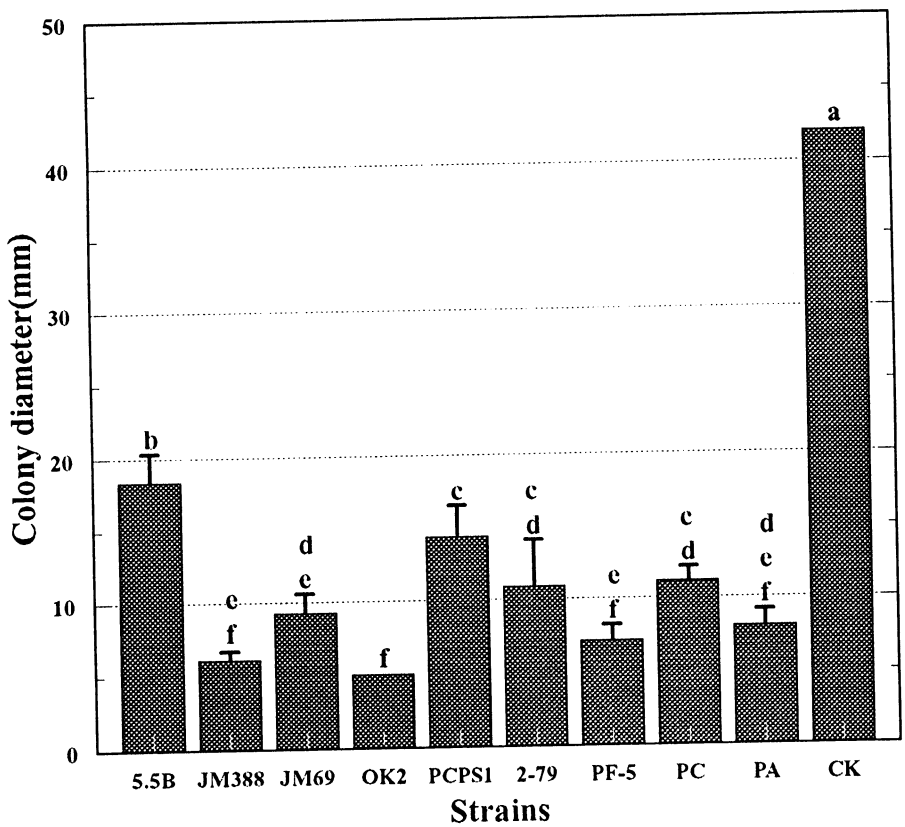

Fig. 1. Inhibition of *Rhizoctonia solani* on potato dextrose agar by different strains of *Pseudomonas* spp. after 3 days. Values are means of data pooled from two experiments. Means are significantly different ($P = 0.05$) if followed by a different letter according to the Waller-Duncan *k*-ratio test. Bars represent standard error of the mean. 5.5B = *P. cepacia,* strain 5.5B; JM388 = *P. cepacia,* strain JM388; JM69 = *P. cepacia,* strain JM69; OK2 = *P. cepacia,* strain OK2; PCPS1 = *P. cepacia,* strain PCPS1; 2-79 = *P. fluorescens,* strain 2-79; PF-5 = *P. fluorescens,* strain PF-5; PC = *P. chlororaphis;* PA = *P. aureofaciens;* CK = *R. solani* growing unchallenged (control).

RESULTS

In vitro inhibition of *R. solani* by Pseudomonas strains. All *Pseudomonas* strains inhibited ($P = 0.05$) *R. solani* in vitro compared with the unchallenged control (Fig. 1). Significantly more inhibition, however, occurred with strain OK2 and JM388 of *P. cepacia,* and strain

PF-5 of *P. fluorescens* compared with several other strains.

Comparison of *Pseudomonas* strains for stem rot control. Only strains of *P. cepacia* were effective ($P = 0.05$) for control of stem rot in polyfoam rooting cubes (Fig. 2). No infection occurred on cuttings in cubes treated with strain 5.5B, strain JM69, strain A, or strain B of *P. cepacia*. In cubes treated with strain JM388, strain OK2, and strain PCPS1 of *P. cepacia*, infection after 14 days was 7, 23, and 93%, respectively. Disease ratings for strains of *P. cepacia* ranged from 1.0 (strain 5.5B, strain JM69, strain A, strain B) to 4.7 (strain PCPS1) (Fig. 2). In comparison, little to no control of stem rot was achieved with strains of *P. fluorescens, P. chlororaphis,* or *P. aureofaciens*. Less ($P = 0.05$) infection occurred on poinsettia cuttings in cubes treated with strain PF-5 of *P. fluorescens* (80% infection) and *P. aureofaciens* (63%), but this was significantly greater than the majority of the strains of *P. cepacia* (Fig. 2). Ratings for disease development on cuttings in cubes treated with strains other than strains of *P. cepacia* ranged from 3.3 (*P. aureofaciens*) to 4.9 (*P. chlororaphis*). Average infection and disease rating for cuttings in the infested control were 97% and 4.7, respectively (Fig. 2). No significant difference in root development occurred with any strain (data not shown).

Comparison of application methods of strain 5.5B of *P. cepacia* for stem rot control. Soaking of rooting cubes with water followed by an over-the-top spray with a suspension of strain 5.5B of *P. cepacia* controlled ($P = 0.05$) Rhizoctonia stem rot in rooting cubes (Fig. 3). Average infection of cuttings in cubes soaked with water followed by an initial bacterial spray at day 0 was 37%. Soaking the rooting cubes in water followed by bacterial sprays at 0, 5, and 10 days after initial soak was the best spray treatment (13% infection). The level of control with this treatment, however, was not significantly different from control achieved by soaking cubes with water followed by sprays at day 0 and 7 (20% infection) or initially soaking rooting cubes with a suspension of strain 5.5B with no additional application of the bacterium (3% infection) (Fig. 3). No infection of cuttings occurred in the noninfested controls, whereas 97% infection occurred in the infested controls (Fig. 3). Disease ratings ranged from 1.1 (bacterial soak alone) to 2.1 (water-soaked cubes followed by one bacterial spray at day 0). Disease ratings on cuttings in controls ranged from 1.0 (noninfested control) to 4.9 (infested control) (Fig. 3). No significant differences in root development occurred on cuttings among any treatments (data not shown).

Soaking the bottom half of cube strips with water and the top half with a

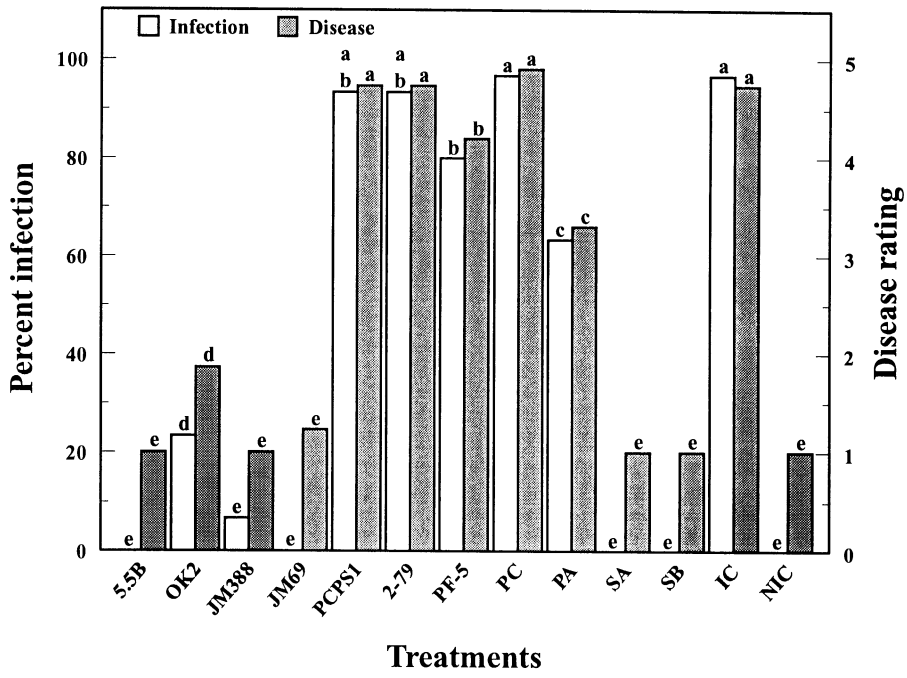

Fig. 2. *Pseudomonas cepacia* (strain 5.5B) compared with other *Pseudomonas* strains for control of Rhizoctonia stem rot of poinsettia in polyfoam rooting cubes. Values are means of data pooled from two experiments. Values within each variable (infection, disease rating) are significantly different ($P = 0.05$) if followed by a different letter according to the Waller-Duncan *k*-ratio test. 5.5B = *P. cepacia,* strain 5.5B; OK2 = *P. cepacia,* strain OK2; JM388 = *P. cepacia,* strain JM388; JM69 = *P. cepacia,* strain JM69; PCPS1 = *P. cepacia,* strain PCPS1; 2-79 = *P. fluorescens,* strain 2-79; PF-5 = *P. fluorescens,* strain PF-5; PC = *P. chlororaphis;* PA = *P. aureofaciens;* SA = *P. cepacia,* strain A; SB = *P. cepacia,* strain B; IC = infested control; NIC = noninfested control.

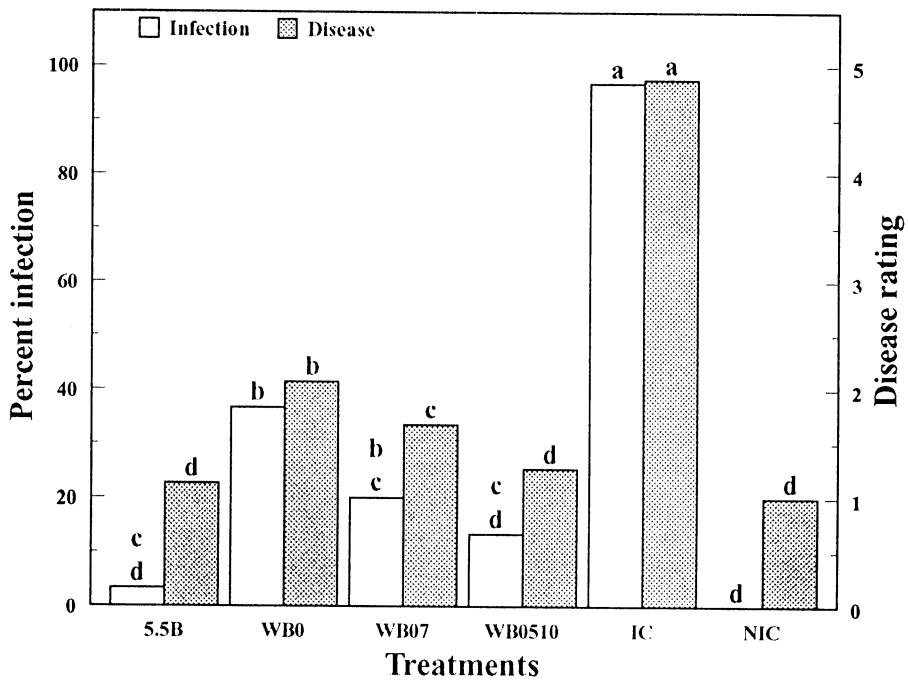

Fig. 3. Efficacy of *Pseudomonas cepacia* (strain 5.5B) in controlling stem rot of poinsettia in polyfoam rooting cubes with different application methods. Values are means of data pooled from two experiments. Values within each variable (infection, disease rating) are significantly different ($P = 0.05$) if followed by a different letter according to the Waller-Duncan *k*-ratio test. 5.5B = cubes soaked in suspension of *P. cepacia,* strain 5.5B, prior to placing cuttings; WB0 = cubes soaked with water then cuttings sprayed over-the-top with suspension of strain 5.5B at day 0; WB07 = cubes soaked with water then cuttings sprayed over-the-top with suspension of strain 5.5B at day 0 and 7 after soaking; WB0510 = cubes soaked with water then cuttings sprayed over-the-top with suspension of strain 5.5B at day 0, 5, and 10 after soaking; IC = infested control; NIC = noninfested control.

suspension of strain 5.5B gave excellent control (3% infection, disease rating = 1.1) of stem rot and was not different ($P = 0.05$) from soaking cube strips with a suspension of strain 5.5B (0% infection) or soaking cube strips with a suspension of strain 5.5B followed by a spray application of strain 5.5B at day 0 (0% infection) (Fig. 4). Dipping stems in a suspension of strain 5.5B prior to placing cuttings in rooting cubes gave no control of stem rot. Spraying poinsettia stock plants with a suspension of strain 5.5B prior to removing and placing cuttings in cubes gave no control ($P = 0.05$) of stem rot compared with the noninfested control (Fig. 4). Disease ratings ranged from 1.0 (5.5B, BB0) to 4.7 (stock plant sprays) (Fig. 4).

DISCUSSION

Many *Pseudomonas* strains possess characteristics considered beneficial for biocontrol agents of soilborne plant pathogens. Among those attributes is the ability to utilize a wide range of nutrient sources, production of inhibitory compounds, and colonization and survival in the target area (6,18,35,36).

In this study, all strains of *Pseudomonas* spp. tested inhibited *R. solani* significantly in vitro. The level of inhibition, however, differed among strains. In other studies, no distinct correlation between in vitro inhibition and efficacy of bacteria to control the target pathogen in vivo existed (14,21). However, the importance of antibiotic production has been demonstrated in some cases by using antibiotic-deficient mutants (31). The *Pseudomonas* strains tested in these experiments differed ($P = 0.05$) in their ability to control stem rot of poinsettia in polyfoam rooting cubes. Only strains of *P. cepacia* gave significant control of stem rot in these tests, with strains 5.5B, A, B, and JM69 of *P. cepacia* giving complete control. Among the strains of *P. cepacia,* only strain PCPS1 failed to control the disease and only very limited control was achieved with strain PF-5 of *P. fluorescens* and the *P. aureofaciens* strain. Because many soilborne microorganisms occupy specific niches, biocontrol agents, depending on their particular attributes, may differ in their effectiveness depending on the system to which they are applied (6,26,36,37). Strain PF-5 and strain 2-79 of *P. fluorescens* have been used successfully to control damping-off of cotton caused by *R. solani* and *Pythium ultimum* and take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici,* respectively (17,31). In addition, both strains produce antifungal metabolites effective against *R. solani.* In this specialized system used for poinsettia, however, little to no control was achieved with these strains. The cube matrix, very liquid absorbent and principally nutrient-free, provides a much different environment than a soil/root system under field conditions, and *P. cepacia* strains seem to possess the characteristics necessary for use under these unusual conditions.

The method of applying biocontrol agents to a target area is critical in the development of biocontrol strategies (7, 15,28). Typically, applying a fungicide to control Rhizoctonia stem rot would involve the use of spray or drench applications to the growing medium (19,30). To date, strain 5.5B has proven very effective when cubes have been soaked with bacterial suspensions prior to placing cuttings. This is more laborious and time consuming, however, than a system of spraying a suspension over the cubes or simply drenching the cubes with a suspension before, or after, cuttings are in place. Likewise, reapplication of the antagonist during the rooting period necessitates the use of a different method(s) of application due to the impracticality of soaking after cube strips are wetted and arranged on benches.

Based on our data, a spray program using strain 5.5B to control Rhizoctonia stem rot in rooting cubes is feasible. The window of application may be more restrictive than using a fungicide but manipulation of carriers or strain improvement could produce more flexibility in a spray program. In addition, an initial soak or drenching of the cubes with strain 5.5B prior to placing cuttings appears to extend the period between applications and provides more effective control. Using a combination of spray and soak/drench applications might be the best approach for developing a long-term control program.

The efficacy of the over-the-top spray and soak/drench treatment to only the top half of the cube demonstrates the critical area of application on the cube for stem rot control. Benson (4) demonstrated that prevention of colonization of rooting cubes by *R. solani* was the crucial factor for effective fungicides. Cartwright and Benson (10) demonstrated that colonization of the top portion of the cubes by *R. solani* was much more rapid than in the bottom or midsections of the cubes. An effective antagonist or preventative compound, therefore, may control *R. solani* if applied to the outer portions of the cube or if it is stable in the outer portions of the cube after application. Over-the-top spraying after soaking cubes in water or soaking the top half of the cube in a suspension of the bacterium demonstrates the importance of coverage in this portion of the cube. Through refinement of methods, it appears that an effective spray program with strain 5.5B to control Rhizoctonia stem rot in rooting cubes can be developed.

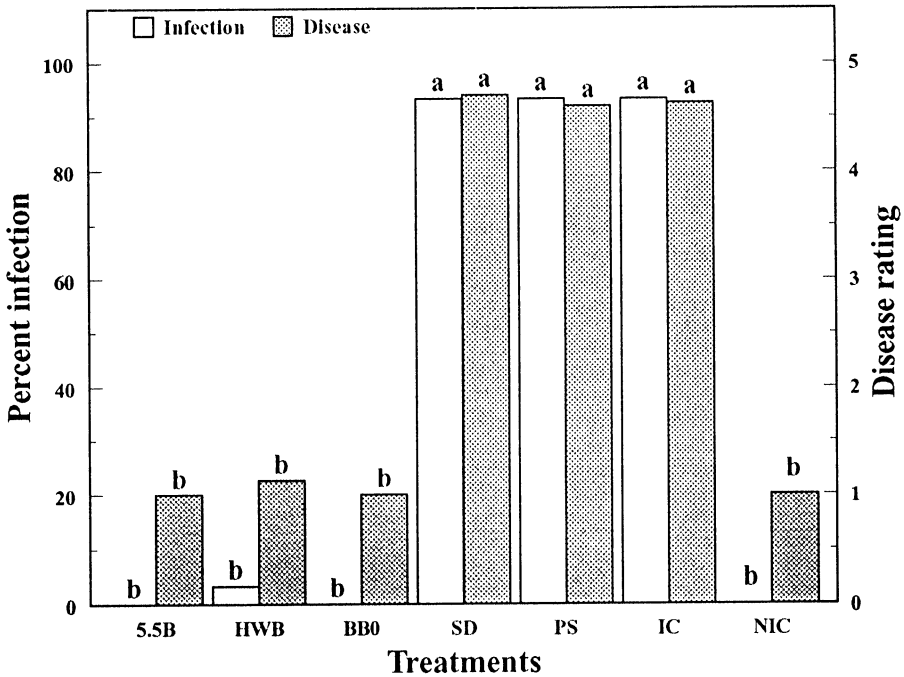

Fig. 4. Efficacy of *Pseudomonas cepacia* (strain 5.5B) in controlling stem rot of poinsettia in polyfoam rooting cubes when applied to rooting cubes, cutting stems, or poinsettia stock plants. Values are means of data pooled from two experiments. Values within each variable (infection, disease rating) are significantly different ($P = 0.05$) if followed by a different letter according to the Waller-Duncan *k*-ratio test. 5.5B = cubes soaked with suspension of *P. cepacia,* strain 5.5B, prior to placing cuttings; HWB = bottom half of cubes soaked with water, top-half sprayed with suspension of strain 5.5B; BB0 = cubes soaked with suspension of strain 5.5B then cuttings sprayed over-the-top with suspension of strain 5.5B at day 0; SD = cutting stems quick-dipped in suspension of strain 5.5B then placed in cubes soaked with water; PS = poinsettia stock plants sprayed with suspension of strain 5.5B prior to taking cuttings that were placed in cubes soaked with water; IC = infested control; NIC = noninfested control.

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