Biocontrol of Damping-off of *Catharanthus roseus* Caused by *Pythium ultimum* with *Trichoderma virens* and Binucleate *Rhizoctonia* Fungi

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ABSTRACT

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Four isolates of *Trichoderma* (*Gliocladium*) *virens* (G-45, G-65, G-85, and G-93) and two isolates of binucleate *Rhizoctonia* spp. (BNR621 and P9023) were evaluated for biocontrol of preemergence damping-off of *Catharanthus roseus* (vinca) caused by *Pythium ultimum*. Putative biocontrol agents were amended to a soilless mix 1, 3, or 6 days prior to seeding and pathogen infestation to determine if colonization of the mix before infestation was important for biocontrol efficacy. Biocontrol of preemergence damping-off of vinca with the four isolates of *T. virens* was variable. Only isolate G-93 gave control of preemergence damping-off (10 to 18% disease) regardless of the length of time the mix was amended prior to seeding and infestation compared to the infested control (43% disease). In contrast, preemergence damping-off was 10 to 15% with SoilGard (based on isolate GL-21 of *T. virens*). For isolate G-65, preemergence damping-off of vinca was 0% in lots of mix amended 1 day prior to seeding, but over 60% in lots of mix amended 6 days prior to seeding, compared to 43% in the infested control. With the exception of isolate G-65 in the lot amended 6 days before seeding, the isolates of *T. virens* were as effective as metalaxyl (19% damping-off) for control of *P. ultimum* in lots of mix amended 1 to 6 days before seeding. In contrast to *T. virens*, biocontrol efficacy of isolates BNR621 and P9023 of binucleate *Rhizoctonia* spp. in a Pesta formulation improved as lots of mix were amended up to 6 days before seeding and infestation. As length of initial amendment increased from 1 to 6 days, preemergence damping-off decreased from 37 to 16% for BNR621, and from 42 to 22% for P9023. Preemergence damping-off was observed in vinca in control treatments with only the putative biocontrol agents (BNR621, 14% disease and P9023, 19.6%); therefore, additional bedding plant species were evaluated for susceptibility to the BNR isolates. In the absence of *P. ultimum*, isolates BNR621 and P9023 in a Pesta formulation caused an average 82.5, 56.5, and 5.8% damping-off of snapdragon, petunia, and impatiens, respectively. Our results suggest that binucleate *Rhizoctonia* isolates, although effective for biocontrol of *P. ultimum* on vinca, should be evaluated for pathogenicity on a crop by crop basis before use on other crops.

Bedding plants account for over 40% of the U.S. floriculture market, which was valued at \$3.93 billion in 1998 (1). Although the wholesale value for other floricultural crops has remained fairly steady for the last 5 years, bedding plant production continues to rise. Typically, bedding plants are grown from seed in plug trays

containing a soilless mix in the greenhouse. Once seedlings are established, the plugs are transplanted to larger containers for finishing. Pathogen-free growing media are used, but damping-off, caused by *Pythium* spp. and *Rhizoctonia solani* Kühn, continues to be a problem after these pathogens become established in greenhouses (24).

The use of biocontrol agents to minimize damping-off is an alternative disease management strategy when growers are concerned over seed phytotoxicity from fungicides. *Trichoderma* (*Gliocladium*) *virens* (J. H. Miller, J. E. Giddens, & A. A. Foster) Arx isolate Gl-21 has been developed into a commercial biocontrol product (SoilGard) for control of *Pythium* and *Rhizoctonia* spp. in ornamental plants, based primarily on studies with one bedding plant species, zinnia (21). Other isolates of *T. virens* have protected agronomic crops, such as cotton, from seedling damping-off (2,3,15,16), but the effectiveness of these isolates for bedding plants is unknown. Gliotoxin and gliovirin, antifungal compounds produced by *T. virens*, were involved in protection of seedlings from *P.*

ultimum Trow (23,26). Howell (16-18), however, has shown that, in addition to antibiosis, a combination of mechanisms was involved in disease suppression by *T. virens*.

Binucleate *Rhizoctonia* fungi (BNR) are another group of biocontrol agents that have proven effective for control of *Pythium* and *Rhizoctonia* spp. diseases on crops such as turf grass (7), potato (11), bean (8), sugar beet (14), cucumber (10,25), pepper and celosia (13), and other bedding plants (12). Colonization of host tissues by nonpathogenic isolates may be an important mechanism for crop protection with BNR (8) that triggers production of host defense compounds such as peroxidases, glucanases, and chitinases (28).

The objective of this research was to evaluate isolates of *T. virens* and binucleate *Rhizoctonia* spp. effective in biocontrol of *Pythium* spp. and *R. solani* on other crops for control of preemergence damping-off in vinca (*Catharanthus roseus* G. Don), one of the most important bedding plant species in the United States. As a result of initial studies on vinca with isolates of BNR in the absence of *Pythium* spp., pathogenicity studies were completed on several additional bedding plant species. Preliminary reports have been presented (5,6)

MATERIALS AND METHODS

Inoculum production. Long-grain rice was pulverized in a Waring blender and sieved through nested sieves with openings of 1.40 and 0.85 mm. Rice particles that passed through the 1.40-mm but not the 0.85-mm sieve were used to establish inoculum. Deionized water (18 ml) was added to 25 g of rice in a 250-ml flask and autoclaved for 25 min on two consecutive days. Isolate Py-59 of *P. ultimum* (H. A. J. Hoitink, Ohio State University, Wooster) from cornmeal agar (CMA) medium was seeded in the flasks by adding three 6-mm-diameter mycelial disks taken from the margin of an actively growing colony on CMA. Rice cultures were incubated for 6 days at ambient temperatures on the laboratory bench. Cultures were removed from the flask and ground lightly with a mortar and pestle and passed through the 1.40- and 0.85-mm sieves again. Inoculum that passed through the 1.40-mm but not the 0.85-mm sieve was used to infest the soilless mix as described below.

***Trichoderma* sp. amendment of soilless mix.** Four 7-mm-diameter potato-dextrose

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This publication reports research involving an antimicrobial substance. It does not contain recommendations for its use, nor does it imply that the uses discussed here have been registered. All uses of pesticides must be registered by appropriate state and federal agencies before they can be recommended.

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agar (PDA) disks of isolates G-45, G-65, G-85, and G93 of *T. virens* (C. Howell, United States Department of Agriculture-Agricultural Research Service, College Station, TX) were added to twice-autoclaved (25 min/day) 5% wheat bran medium (25 cm³ wheat bran per 500 ml of deionized water). Cultures were incubated for 7 days on a shaker (Thermolyne, Dubuque, IA) at 200 rpm. Liquid cultures were filtered through a 1-mm screen and the retentate dried in a laminar flow hood for 24 to 36 h. The wheat bran preparation, consisting mostly of chlamydospores, was removed from the screen after drying and passed through a 2-mm sieve, then stored at 4°C until needed.

The wheat bran preparation for each *Trichoderma* isolate was added at a rate of 3 cm³ of preparation per 1,000 cm³ (0.3% vol/vol) of Fafard no. 2 soilless mix (Fafard Inc., Agawam, MA) at 65% moisture. Individual lots of amended mix were rotated and shaken for 2 min in plastic bags. Mix was amended with each isolate 1, 3, or 6 days prior to seeding. Lots of mix amended 6 and 3 days prior to seeding were incubated in bags. All lots were used to fill plug trays (81-cell, each cell 2.5 by 2.5 cm, tapered to 2.5 cm in depth) 1 day prior to seeding. Filled trays were placed in a mist system that cycled on for 2 min, five times daily. A portion of each lot of amended mix was set aside in bags in the greenhouse for use as a seed covering as described below.

Pesta formulation of BNR. Binucleate *Rhizoctonia* isolates BNR621 (formerly 232-CG) and P9023 from the collection of the late E. Echandi (North Carolina State University) were formulated by a modified Pesta process developed by Connick et al. (9). Four 7-mm-diameter disks of each isolate taken from the margins of cultures actively growing on PDA were seeded in 50 g (1:1 wt/vol) of autoclaved wheat bran and deionized water in a 1-liter flask and grown for 7 days at room temperature. The resulting wheat bran culture was collected on cheesecloth lining a Buchner funnel. The culture was then transferred to a weigh boat and brought to 160 g with deionized water. This additional water resulted in a workable dough when the moistened wheat bran was mixed by hand with 160 g of semolina and 40 g of kaolin clay (20% fungal biomass, wt/wt). The dough was kneaded with gloved hands into a ball and then rolled into sheets, 3 to 5 mm thick. The sheets were dried on a fiberglass screen rack under sterile air from a laminar flow hood for 24 h, broken into small pieces, briefly pulverized in a Waring blender, screened through a sieve with 2-mm openings, and then stored at 4°C until used.

BNR amendment of soilless mix. At 1, 3, or 6 days prior to seeding 81-cell plug trays as described below, 3.5 g of Pesta granules of isolate BNR621 or P9023 were amended to 1,000 cm³ of Fafard no. 2

soilless mix (1% vol/vol) in black plastic bags. The moisture content of the mix was adjusted to approximately 65% (wt/wt). The bags were inflated partially, then rotated for 2 min to distribute the granules throughout the mix. Bags were stored in the greenhouse at 22 to 27°C to allow initial colonization of the mix by the BNR isolates. Plug trays were filled with the amended mix 1 day prior to seeding and placed under the mist system. Sufficient mix was reserved in bags for each treatment to cover seeds after plug trays were seeded.

Biocontrol experiments. Plug trays filled with lots of the various amended mixes, or nonamended mix described below, and were vacuum seeded (Berry Seeder Co., Elizabeth City, NC) with vinca 'Peppermint Cooler' (Ball Horticultural Co., West Chicago, IL). Seeds in each tray were covered by sprinkling 150 cm³ of reserved mix infested with 0.5 g of rice particles of *P. ultimum* from a 500-cm³ bottle with seven 8-mm-diameter holes in the lid. In preliminary experiments, this amount of *Pythium* sp. inoculum caused 50 to 80% preemergence damping-off. Controls included infested, nonamended potting mix; noninfested, nonamended potting mix; SoilGard 12 G (based on *T. virens* isolate GI-21) at a label rate of 4.5/1,000 cm³; and metalaxyl (Subdue 2E, Novartis Crop Protection, Inc., Greensboro, NC) at a label rate of 0.01 ml a.i./liter. Metalaxyl was applied in a drench in a volume of 325 ml per plug tray immediately after seeding and infesting the mix with *P. ultimum*. Three replicates of each treatment were arranged in a completely randomized design on a greenhouse bench under the mist system. Stand counts were made until maximum emergence occurred in the noninfested, nonamended control. The germination rate for vinca seeds is about 70%. Preemergence damping-off in the various treatments was based on stand count in the noninfested, nonamended control.

Pathogenicity tests of BNR isolates. Results of preliminary tests suggested that the BNR isolates caused damping-off of vinca and snapdragon (*Antirrhinum majus*). Therefore, additional experiments were conducted with impatiens (*Impatiens wallerana* 'Super Elfin Mix'), and petunia (*Petunia × hybrida* 'Madness Series') as well as snapdragon 'Sprite Mix.' Plug trays were filled 1 day prior to seeding with Fafard no. 2 mix that had been amended with 3.5 g of Pesta formulation of either isolate BNR621 or P9023 per 1,000 cm³ of mix (1% vol/vol). Test parameters were as described above for biocontrol experiments. Postemergence damping-off occurred in addition to preemergence damping-off with some bedding plant crops; therefore, damping-off percentage was based on stand count at 4 weeks.

Statistical design and analysis. All experiments were conducted at least twice.

Experimental data were pooled when possible based on homogeneity of variances. Data for biocontrol experiments with BNR isolates and BNR pathogenicity tests were analyzed with PROC GLM (SAS Institute, Cary, NC) and means were separated by a Waller-Duncan K-ratio *t* test. Data from biocontrol experiments with isolates of *T. virens* were analyzed with PROC MIXED. Least significant means were separated by linear contrasts by use of the PDIF option.

RESULTS

Biocontrol of *P. ultimum* with *T. virens*. Preemergence damping-off of vinca was significantly less ($P = 0.05$) in lots of soilless mix amended with isolate G-65, G-85, or G-93 1 day before seeding and infesting with *P. ultimum*, compared to 43% damping-off observed in the nonamended, infested control (Fig. 1A). Control of preemergence damping-off with isolates G-65, G-85, or G-93 was not different ($P = 0.05$) than control observed with metalaxyl or SoilGard (19 and 11% damping-off, respectively; Fig. 1A). Preemergence damping-off of vinca seeded in mix that had been amended with isolate G-45 or G-93 3 days before seeding and infesting was significantly less ($P = 0.05$) than that observed in the nonamended, infested control (Fig. 1B). No difference in damping-off percentage was observed ($P = 0.05$) between isolates G-45 or G-93 and metalaxyl or SoilGard for vinca in lots of mix amended 3 days before seeding. Preemergence damping-off of vinca was significantly less ($P = 0.05$) only in the lots of mix amended with isolate G-93 (17% damping-off) or SoilGard (15% damping-off) 6 days before seeding compared to the infested, nonamended control (43% damping-off, Fig. 1C). Damping-off of vinca averaged 62% in lots of mix with isolate G-65 amended 6 days before seeding and infesting.

Biocontrol of *P. ultimum* with BNR. Vinca in lots of mix amended 6 days before seeding with Pesta formulations of BNR isolates had less preemergence damping-off than vinca in lots amended 1 or 3 days before seeding and infesting with *P. ultimum* (Fig. 2A to C). Preemergence damping-off of vinca was significantly less ($P = 0.05$) in mix amended with Pesta formulations of BNR621 or P9023 (38 and 42% damping-off, respectively) 1 day prior to seeding and infesting, compared to the infested, nonamended control (58% damping-off, Fig. 2A). Vinca in lots of mix amended with either BNR isolate 3 or 6 days before seeding and infesting with *P. ultimum* had less ($P = 0.05$) damping-off than vinca in the infested, nonamended control (Fig. 2B and C). Preemergence damping-off of vinca was least in lots of mix with Pesta formulations of isolates BNR621 and P9023 (15 and 22% damping-off, respectively)

amended 6 days before seeding and infesting (Fig. 2C).

In the absence of *P. ultimum*, vinca in lots of mix amended with Pesta formulations of isolate P9023 either 1 or 3 days before seeding had more ($P = 0.05$) damping-off (22 and 25%, respectively) than the noninfested control (0% damping-off, Fig. 2A and B). Preemergence damping-off of vinca in lots amended with the Pesta formulation of isolate BNR621 at 1, 3, or 6 days before seeding was not different than the noninfested control (Fig. 2A to C). Damping-off of vinca averaged 14 and

19.6% for Pesta formulations of isolates BNR621 and P9023, respectively, across lots of mix amended 1, 3, and 6 days before seeding.

Pathogenicity of BNR isolates. Pesta formulations of isolates BNR621 and P9023 caused an average of 5.8% damping-off for impatiens, 82.5% for snapdragon, and 56.5% for petunia over two experiments (Fig. 3A to C). Differences between isolates were not significant except on petunia, where isolate P9023 caused more damping-off than BNR621 (Fig. 3C). Both preemergence and poste-

mergence damping-off was observed over a 4-week period after seeding. Postemergence damping-off occurred most frequently on snapdragon and petunia.

DISCUSSION

In general, the length of time isolates of *T. vires* were amended to the soilless mix before seeding and infesting did not affect the extent of biocontrol of *P. ultimum* observed. However, with isolate G-65, biocontrol ability was less as length of time between amending lots of mix and seeding increased. Isolate G-93 gave the best biocontrol of *P. ultimum* regardless of whether the mix was amended 1, 3, or 6 days before seeding and infesting. Isolates G-65 and G-85 controlled preemergence damping-off of vinca in lots of mix amended 1 day before seeding and infesting, but longer periods were not effective. Isolate G-45 was inconsistent in biocontrol of *P. ultimum* because preemergence damping-off was less than the infested, nonamended control in lots of mix amended 3 days before seeding, but not in lots of mix amended 1 or 6 days before seeding. Although isolate 93 gave the best biocontrol of *P. ultimum* of the *T. vires* isolates tested, over the three amendment periods, preemergence damping-off of vinca averaged 15.3%, which may be unacceptable disease control for commercial growers. However, pre-emergence damping-off of vinca was 19% with metalaxyl, so biocontrol with isolate G-93 was comparable to control of pre-emergence damping-off achieved with a standard fungicide in our studies.

In contrast to the inconsistency of isolates G-43, G-65, and G-85, SoilGard was consistent in control of *Pythium* pre-emergence damping-off of vinca regardless of whether the mix was amended 1, 3, or 6 days before seeding and infesting. SoilGard was one of the first fungal-based biocontrol products registered by the Environmental Protection Agency in the United States (20,22). Although Lumsden and Locke (20) and Lewis et al. (19) found that lots of mix amended 7 days before seeding with isolate G1-21 (SoilGard) were the most effective for control of pre-emergence damping-off of zinnia caused by *P. ultimum*, the SoilGard label recommends amending the mix 1 day before seeding. In lots of mix amended with SoilGard 1 day before seeding and infesting with *P. ultimum*, we found about 10% pre-emergence damping-off of vinca, an incidence of disease that may be unacceptable to commercial growers. Increasing the time between amending lots of mix and seeding did not improve disease control with SoilGard.

Isolate G-65 was different from the other isolates of *T. vires* tested for biocontrol against *P. ultimum*. The efficacy of G-65 changed dramatically, from 0% pre-emergence damping-off of vinca in lots of mix amended 1 day before seeding and infesting to 30 and 62% damping-off in

Fig. 1. Preemergence damping-off of vinca (*Catharanthus roseus*) caused by *Pythium ultimum* after amendment of soilless mix with isolates of *Trichoderma vires*. (A) Mix amended 1 day before seeding and infesting with *P. ultimum*, (B) mix amended 3 days before seeding and infesting, and (C) mix amended 6 days before seeding and infesting. Values are least square means of data pooled from three experiments and means are significantly different ($P = 0.05$) if followed by a different letter. Treatments were C = noninfested, nonamended control, IC = infested, nonamended control, M = metalaxyl, SG = SoilGard, 45 = isolate G-45, 65 = isolate G-65, 85 = isolate G-85, and 93 = isolate G-93 of *T. vires*.

lots of mix amended 3 and 6 days, respectively, before seeding and infesting. Isolate G-65 is classified in the "P" group of *T. virens* that primarily produces gliovirin, while isolates G-45, G-85, and G-93 are "Q" group isolates that produce gliotoxin (C. Howell, *personal communication*).

Gliovirin was inhibitory to *P. ultimum* in experiments with gliotoxin minus mutants (18), but gliovirin has been studied less than gliotoxin for persistence once culture preparations are added to soil. When gliotoxin produced by isolate GL-21

(SoilGard) was extracted from a soilless medium over an 18-day period, the maximum amount of gliotoxin peaked between 1 and 4 days, then diminished rapidly between 9 and 18 days (20). Gliovirin production may behave the same way, explaining the reduction in biocontrol efficacy of G-65 over time. That is, G-65 failed to produce gliovirin in the mix during the 6-day period following amendment and, by the time the mix was seeded with vinca, the gliovirin produced in the wheat bran culture of G-65 had diminished below

Fig. 2. Preemergence damping-off of vinca (*Catharanthus roseus*) caused by *Pythium ultimum* after amendment of soilless mix with isolates of binucleate *Rhizoctonia* spp. in a Pesta formulation. (A) Mix amended 1 day before seeding and infesting with *P. ultimum*, (B) mix amended 3 days before seeding and infesting, and (C) mix amended 6 days before seeding and infesting. Values are significantly different ($P = 0.05$) according to the Waller-Duncan k-ratio, t test, $k = 100$, if followed by a different letter. Treatments were C = noninfested, nonamended control, IC = infested, nonamended control, B = Pesta formulation of isolate BNR621 in the absence of *P. ultimum*, UB = Pesta formulation of isolate BNR621 in mix infested with *P. ultimum*, P = Pesta formulation of isolate P9023 in the absence of *P. ultimum*, and UP = Pesta formulation of isolate P9023 in mix infested with *P. ultimum*.

Fig. 3. Percent damping-off after 4 weeks of (A) impatiens (*Impatiens wallerana*), (B) snapdragon (*Antirrhinum majus*), and (C) petunia (*Petunia × hybrida*), grown in a potting mix amended 1 day before seeding with a Pesta formulation of isolate BNR621 or P9023 of binucleate *Rhizoctonia* spp. Values followed by a different letter within experiment I or II are significantly different ($P = 0.05$) according to the Waller-Duncan k-ratio, t test, $k = 100$.

an effective concentration. Production of viridiol, a compound with herbicidal activity that is produced by some strains of *T. virens*, may have accounted for the increase in preemergence damping-off observed in mix amended with isolate G-65 3 and 6 days before seeding. However, Wilhite et al. (26) found *T. virens* mutants that overproduced viridin and, subsequently, viridiol did not reduce seedling stands of zinnia in soilless mix. The sensitivity of vinca to viridiol is unknown. Attributing the observed decrease in efficacy to a reduction in the population of G-65 is less likely. In studies conducted by Lewis et al. (19), as well as Wolffhechel and Jensen (27), neither 100-fold nor 1,000-fold differences in inoculum density affected the efficacy of different *T. virens* isolates tested for biocontrol of *P. ultimum* (19,27).

Both isolates of binucleate *Rhizoctonia* spp. tested controlled preemergence damping-off of vinca caused by *P. ultimum* compared to the infested control. Efficacy of the two isolates was best when lots of mix were amended 6 days before seeding and infestation with *P. ultimum*. Presumably, mycelium from the Pesta formulation colonized the soilless mix during the 6-day period between amendment and seeding of the mix and, therefore, protected vinca seeds from *P. ultimum*. In another study with isolates of BNR, Harris et al. (13) reported control of *P. ultimum* var. *sporangiferum* on celosia and *R. solani* on other bedding plants (12). No detrimental effects of the BNR isolates were found on seed germination and emergence (12).

Isolates BNR621 and P9023 were pathogenic to snapdragon, petunia, and vinca in our studies, while damping-off of impatiens was less than 6%, depending on isolate. Cubeta and Echandi (10) found that BNR621 and P9023 were not pathogenic on bean or potatoes. In our studies, we did observe necrotic areas on bean hypocotyls with these isolates (J. R. Burns, unpublished data), while Benson and Barker (4) observed limited necrosis on cotton hypocotyls with these isolates.

Efficacy of isolates of *T. virens* tested for biocontrol of *P. ultimum* did not improve as time between amendment of soilless mix and seeding increased, while preemergence damping-off of vinca was least in lots of mix amended with Pesta formulations of binucleate *Rhizoctonia* spp. 6 days before seeding and infesting. In commercial production of bedding plants, a 1-day period between filling and moistening the mix in plug trays, followed by seeding the next day, is typical of production practices. Isolates of *T. virens* should perform well under these conditions. On the other hand, isolates of BNR fungi

would not be compatible with production facilities that could not allow trays with potting mix to stand 6 days before seeding; therefore, manipulation of formulation or selection of more effective strains may be needed before BNR fungi are adopted as biocontrol agents for *P. ultimum*.

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