Beneficial Bacteria Identified for the Control of *Botrytis cinerea* in Petunia Greenhouse Production

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Abstract

Botrytis cinerea infects most major greenhouse crops worldwide. With its increasing resistance to conventional fungicides and the movement of the greenhouse industry toward more sustainable production practices, alternative methods of control are needed. The objective of this study was to evaluate a collection of 60 bacterial strains through both a dual-culture assay and greenhouse trials to identify strains with biocontrol activity against *B. cinerea*. For the dual-culture assay, each bacterial strain was streaked on potato dextrose agar medium with *B. cinerea*. The *B. cinerea* growth reduction and the zone of inhibition were measured. Thirty-five strains reduced the growth of *B. cinerea*. All strains were also tested in an initial greenhouse trial in which *Petunia × hybrida* ‘Carpet Red Bright’ was sprayed and drenched with the bacteria biweekly for 6 weeks. All open flowers were tagged, and plants were inoculated with *B. cinerea* (1×10^4 conidia per 1 ml). Disease

severity indices calculated from the daily flower gray mold severity ratings of all tagged flowers were used to identify the seven top-performing strains. These seven strains were then evaluated in a greenhouse validation trial. The methods were similar to those of the initial greenhouse trials except that replicate numbers were increased. Three strains (*Pseudomonas protegens* AP54, *Pseudomonas chlororaphis* 14B11, and *Pseudomonas fluorescens* 89F1) were selected for the ability to reduce *B. cinerea* infection in a greenhouse production setting. These strains can be used in future studies to develop additional biocontrol products for the management of *B. cinerea* in floriculture crops.

Keywords: bacteria, biocontrol, botrytis blight, floriculture, fungus, gray mold, *Pseudomonas*

Botrytis cinerea, the causal agent of gray mold, is one of the most common and economically devastating pathogens of greenhouse crops because of its wide host range and ability to infect plants at all stages of the production chain (Dean et al. 2012; Dik and Wubben 2007). The production of flowering bedding plants is particularly problematic because of the high susceptibility of the flowers to gray mold. Infected flowers reduce crop quality, and in severe cases, they can lead to crop loss (Dik and Wubben 2007). The greenhouse environment in early spring can provide ideal conditions for *B. cinerea* to infect plants. Cool temperatures, high humidity, reduced air movement, and wet flowers and leaves are conducive to *B. cinerea* infection (Dik and Wubben 2007).

Increasing air temperatures and ventilation and decreasing relative humidity can be effective ways to reduce *B. cinerea* infection, but these environmental manipulations may not be feasible for some greenhouse production facilities (Dik and Wubben 2007). Fungicides are commonly used to manage this pathogen, but resistance to several active ingredients has been reported in various geographical regions (Fungicide Resistance Action Committee 2018) for field crops (Cosseboom et al. 2019) and greenhouse crops (Muñoz et al. 2019). The emergence of resistance to chemical fungicides coupled

with increased customer demands for plants free of pesticide residues have created a need for additional management options (Lamichhane et al. 2016; Pal and McSpadden Gardener 2006; Xia et al. 2006).

Biological control or biocontrol of plant pathogens is an alternative method of management that uses organisms that are introduced or already established in a system (Pal and McSpadden Gardener 2006). Beneficial plant–microbe interactions have been used throughout history for controlling diseases in trees, row crops, and greenhouse crops caused by both soilborne and foliar pathogens (Cook and Baker 1983; Fravel 2005). The greenhouse offers a unique opportunity to study and implement biocontrol of plant pathogens owing to the level of environmental control and the type of crops grown (Paulitz and Bélanger 2001). There is an increasing interest in understanding how biocontrol can be used as an additional tool in integrated pest management programs for many pathogens (Pal and McSpadden Gardener 2006; Syed Ab Rahman et al. 2018), including *B. cinerea* (Elad and Steward 2007). As a result, biopesticides are currently the fastest-growing sector of the crop protection market, and microbial biopesticides represent the largest use segment of the biopesticide industry (Meister Media Worldwide 2014).

Studies have shown that bacteria have the potential to control *B. cinerea* in crops, such as grapevine (Barka et al. 2002), tomato (Yildiz et al. 2007), and petunia (Gould et al. 1996). Promising bacterial strains are typically identified by isolation from either a different plant system (e.g., an isolate from onion root evaluated in grapevine) (Barka et al. 2002) or the same plant system (e.g., isolates from tomato leaves evaluated in tomato plants) (Yildiz et al. 2007). Research on the biocontrol of gray mold has been conducted with greenhouse floriculture crops. A yeast and a bacterium isolated from rosebuds were found to reduce lesions caused by *B. cinerea* on whole cut roses by 63 and 48%, respectively, whereas iprodione-treated roses had a 74% reduction in lesions (Redmond et al. 1987). A *Pseudomonas fluorescens* strain that reduced the incidence of gray mold was identified from a collection of 172 bacterial strains isolated from petunia tissues. When applied to petunia flowers on plants in an incubation chamber, disease incidence was reduced by an average of 77% (Gould et al. 1996).

Studies evaluating large bacteria collections to identify potential biocontrol agents commonly use an in vitro method to narrow down

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the candidates before in planta studies are conducted. In vitro screening efforts can provide valuable information, such as an indication of mode of action, but they seldom accurately predict what is observed in in planta studies. The complex interactions between the introduced microbe, plant, environment, and pathogen can be most accurately evaluated using in planta studies (Köhl et al. 2019; Pal and McSpadden Gardener 2006). Milus and Rothrock (1997) identified top-performing strains in an in vitro assay looking at inhibition zone width for the potential control of *Pythium* spp., but these strains were not as effective as others in the field. In light of these and other similar results, it is becoming more common to evaluate potential beneficial microbes in the system for which they are being studied (Fravel 2005).

The overall goal of this study was to identify bacteria from a collection of 60 bacterial strains for the potential to control the fungal pathogen *B. cinerea* in greenhouse bedding plant production. The specific objectives were (i) to identify bacterial strains that can reduce *B. cinerea* growth through a dual-culture assay and (ii) to identify bacterial strains that can reduce gray mold severity in planta using greenhouse trials.

Materials and Methods

Dual-culture assay. Forty-five bacterial strains from Dr. Christopher Taylor (The Ohio State University, Wooster, OH) previously described by Subedi et al. (2019) along with 15 strains from Dr. Joseph Klopper (Auburn University, Auburn, AL) were tested for the ability to inhibit the growth of *B. cinerea* in a dual-culture assay adapted from Wang et al. (2009) (Table 1). Bacterial strains were grown from -80°C glycerol storage on LB agar for 24 h at 28°C . Bacteria were resuspended in Luria-Bertani (LB) broth, measured for optical density (OD) at 595 nm on a spectrophotometer (DTX880; Beckman Coulter, Brea, CA), and adjusted to an OD_{595} of 0.2. The *B. cinerea* isolate acquired from the Application Technology Research group (U.S. Department of Agriculture/Agricultural Research Service, Wooster, OH) was originally isolated from annual begonias, and its identity was confirmed by PCR with a primer set specific to *B. cinerea* (Rigotti et al. 2002). For the dual-culture assay, a single bacterial strain and *B. cinerea* were cocultured on potato dextrose agar (PDA). A 5-mm-diameter cork borer was used to cut a mycelial plug from the edge of a 3-day-old *B. cinerea* culture on PDA. The plug was placed in the center of a 100-mm petri dish containing approximately 25 ml of PDA (Fig. 1). On two opposite sides of the plug, 10 μl of bacterial suspension was streaked in a straight line 2.5 cm away from the plug so that the bacterial streaks were parallel to each other (Fig. 1). Ten microliters of sterile LB broth were used as the negative control. For a positive control, three sterile 6-mm filter paper disks were placed in lines on each side of the plug 2.5 cm from the center, and 5 μl of 58.6 mM cycloheximide (water:ethanol, 1:1 vol/vol) was added to each disk. Petri dishes were sealed with parafilm and incubated at 20°C (I-35 VL; Percival Scientific Inc., Boone, IA). Each treatment was replicated three times and arranged in a randomized complete block design inside the incubator.

When the mycelial growth in the negative control treatment reached the edges of the petri dish, measurements were taken for all treatments. The growth of *B. cinerea* was measured from the edge of the agar plug to the leading edge of the mycelial growth parallel (R1) and perpendicular (R2) to the bacterial streak (Fig. 1). These measurements were taken for each of the bacterial streaks on the plate for a total of four growth measurements per petri dish. The average of R1 and R2 measurements were used to calculate the percentage growth reduction compared with the negative control according to the following formulas:

$$\text{R1 Growth Reduction (\%)} = \frac{(R1_{\text{control}} - R1_{\text{Dual Plate}})}{R1_{\text{control}}} \times 100$$

$$\text{R2 Growth Reduction (\%)} = \frac{(R2_{\text{control}} - R2_{\text{Dual Plate}})}{R2_{\text{control}}} \times 100$$

A zone of inhibition, defined as the distance from the leading edge of bacteria growth to that of *B. cinerea* mycelium (R3), was also

measured on each side of the plug (Fig. 1), and measurements were averaged for each plate.

Initial greenhouse trial for bacterial strain selection. *Experimental design.* The initial greenhouse trial was conducted using an augmented design (Federer and Raghavarao 1975). All 60 bacterial strains from the collection were split into three experimental groups and evaluated for their ability to control gray mold. A commercial biocontrol product containing *Bacillus subtilis* QST 713 (Cease; BioWorks, Victor, NY) served as the positive control, whereas the negative control was sterile LB medium. There were three replicates of each bacterial strain, and both the positive and negative controls were overreplicated, with 12 replicates in each experimental group.

Plant material. *Petunia* $\times$ *hybrida* ‘Carpet Red Bright’ seeds (Ivy Garth Seeds & Plants, Chesterland, OH) were sown in soilless substrate (Promix PGX; Premier Tech, Quebec, Canada) in plug trays and grown under fluorescent lighting for 4 weeks. Plugs were transplanted into 11.4-cm plastic pots into soilless substrate (Baccto Bark 1000 Professional Planting Mix; Michigan Peat Company, Houston, TX) and moved to the greenhouse. Day temperature was maintained between 21 and 24°C , and night temperature was between 16 and 18°C with a 14-h photoperiod. Light levels were maintained at 300 W/m^2 using supplemental lighting. Petunias were irrigated as needed with 50 mg/liter of N from 15 N:2.2 P:12.5 K:2.9 Ca:1.2 Mg water-soluble fertilizer (JR Peters, Allentown, PA).

Bacterial treatment of plants. All treatments were applied at transplant and every 2 weeks thereafter for a total of four applications. Bacterial treatments were prepared by growing strains on LB agar from glycerol storage for 36 h at 28°C . Single colonies were selected and grown in LB broth for 9 h at 28°C on an orbital shaker at 200 rpm. Optical density was measured at 600 nm using a spectrophotometer (UV-1600PC; VWR International, LLC, Radnor, PA) and adjusted with sterile LB broth to an OD_{600} of 0.8. Bacterial cultures were diluted 1:100 with reverse osmosis (RO) H_2O . The positive control (Cease; BioWorks) was applied at the recommended rate of 15 ml/liter. At transplant, 80 ml of each treatment was applied as a drench to each pot. For the second and third applications, 80 ml of each treatment was sprayed using handheld plastic trigger spray bottles onto the leaves, flowers, and potting substrate. For the final application, treatments were applied as a spray (40 ml) to the leaves and flowers and as a drench (80 ml) to the potting substrate.

Pathogen inoculation. After the final treatment application, the plants were moved into a greenhouse environment more conducive for *B. cinerea* infection. Plants were placed under tents created by draping clear greenhouse plastic over PVC pipe structures on each of three greenhouse benches. Inside each tent, an automated fogging system (Trident Single Zone Controller Model T3A; Phytotronics, Inc., Earth City, MO) was used to increase humidity and maintain water droplets on the plants, fogging for approximately 8 s every 30 min. The greenhouse settings were adjusted to a day temperature between 18.3 and 21.1°C and a night temperature between 15.6 and 18.3°C with reduced light levels, and the horizontal airflow fans were turned off. Plants were irrigated with 50 mg/liter of N from 15 N:2.2 P:12.5 K:2.9 Ca:1.2 Mg water-soluble fertilizer (JR Peters) for 1 min twice a day through drip irrigation. Before *B. cinerea* inoculation, all open flowers on each plant were tagged for identification and subsequent rating. *B. cinerea* was grown on PDA at 20°C in darkness for at least 10 days. Conidia were collected by flooding plates with sterile nanopore H_2O with Tween 20 (0.5% vol/vol), and conidia concentration was determined by hemocytometer counts. Conidia were then added to RO water to a final concentration of 1×10^4 conidia per 1 ml, and a hand pressure sprayer was used to deliver approximately 50 ml of the spore suspension to each plant.

Disease observations. Daily disease severity rating began when symptoms were first observed on the flowers, 48 h after *B. cinerea* inoculation, and continued until 2 weeks after the last treatment application using a 0 to 7 rating scale developed in preliminary trials (Table 2). The daily flower ratings were used to calculate an index of disease (ID) using the following formula, where n_x is the number of flowers with a particular severity rating (x) per plant and N is the

Table 1. Collection of 60 beneficial bacteria evaluated for *Botrytis cinerea* control in a dual-culture assay and greenhouse trials

Bacterial strain	Bacterial name ^a	Source	Dual-culture assay ^b			Initial ^f greenhouse trial: Overall disease severity score ^g
			R1 GR (%) ^c	R2 GR (%) ^d	R3 (mm) ^e	
12H11	<i>Pseudomonas protegens</i>	Missouri River	41.35 ± 1.12	71.31 ± 0.42	2.5 ± 0	2
14B11	<i>Pseudomonas chlororaphis</i>	Missouri River	26.44 ± 3.66	68.48 ± 0.13	3.33 ± 0.6	36
14B2	<i>Pseudomonas protegens</i>	Missouri River	45.57 ± 0.73	70.46 ± 0.84	4 ± 0	5
14D6	<i>Pseudomonas chlororaphis</i>	Mississippi River	14.77 ± 4.15	32.49 ± 3.45	0 ± 0	0
MBSA-MJ1 ^h	<i>Serratia plymuthica</i>	Unknown	31.64 ± 3.8	62.45 ± 1.84	0 ± 0	3
15G2	<i>Pseudomonas protegens</i>	Missouri River	40.51 ± 1.93	70.46 ± 1.52	2.67 ± 0.33	0
15H3	<i>Pseudomonas protegens</i>	Missouri River	37.42 ± 1.24	70.23 ± 0.98	3.67 ± 0.33	26
1B1	<i>Pseudomonas protegens</i>	Mississippi River	28.32 ± 4.97	64.85 ± 2.5	2.83 ± 0.44	0
1C5	<i>Pseudomonas protegens</i>	Mississippi River	22.78 ± 4.38	43.46 ± 7.81	0 ± 0	0
24D3	<i>Pseudomonas fluorescens</i>	Herbarium	17.3 ± 1.12	59.49 ± 1.46	0 ± 0	6
28B5	<i>Pseudomonas fluorescens</i>	Herbarium	13.08 ± 4.02	59.92 ± 3.61	0 ± 0	2
29G9	<i>Pseudomonas poae</i>	Herbarium	7.35 ± 7.84	73.69 ± 2.13	4.33 ± 0.17	0
2F9	<i>Pseudomonas fluorescens</i>	Missouri River	34.18 ± 3.19	64.14 ± 0.42	0 ± 0	4
36B3	<i>Pseudomonas fluorescens</i>	Wyoming soil	11.33 ± 5.97	39.49 ± 3.66	0 ± 0	0
36B7	<i>Pseudomonas brassicacearum</i>	Wyoming soil	16.98 ± 8.44	71.03 ± 1.95	4 ± 0.29	0
36C6	<i>Pseudomonas frederiksbergensis</i>	Wyoming soil	16.3 ± 7.47	56.39 ± 1.84	0 ± 0	14
36C8	<i>Pseudomonas poae</i>	Wyoming soil	10.65 ± 0.9	46.79 ± 4.83	0 ± 0	2
36D4	<i>Pseudomonas brassicacearum</i>	Wyoming soil	10.37 ± 3.15	70.18 ± 1.58	4.25 ± 0.38	0
36F3	<i>Pseudomonas fluorescens</i>	Wyoming soil	31.22 ± 5.68	63.29 ± 0.73	0.67 ± 0.67	18
36G2	<i>Pseudomonas fluorescens</i>	Wyoming soil	19.83 ± 2.57	57.81 ± 0.42	0 ± 0	6
37A10	<i>Pseudomonas frederiksbergensis</i>	Wyoming soil	18.99 ± 4.07	69.2 ± 0.85	2.5 ± 0	14
37D10	<i>Pseudomonas brassicacearum</i>	Wyoming soil	2.11 ± 1.52	25.63 ± 0.48	0 ± 0	3
38D4	<i>Pseudomonas brassicacearum</i>	Wyoming soil	16.88 ± 1.84	33.75 ± 0.84	0 ± 0	21
38D7	<i>Pseudomonas brassicacearum</i>	Wyoming soil	5.07 ± 4.44	27.46 ± 3.26	0 ± 0	0
38F7	<i>Pseudomonas frederiksbergensis</i>	Wyoming soil	13.92 ± 2.63	59.49 ± 1.93	0 ± 0	1
38G2	<i>Pseudomonas protegens</i>	Wyoming soil	28.22 ± 1.63	69.51 ± 0.41	3.17 ± 0.33	23
39A2	<i>Pseudomonas frederiksbergensis</i>	Wyoming soil	9.96 ± 2.12	67.25 ± 0.61	3 ± 0.29	7
48B8	<i>Pseudomonas chlororaphis</i>	Wyoming soil	24.78 ± 0.73	67.22 ± 0.85	3.17 ± 0.17	0
MBSA-3BB1 ^h	<i>Pantoea agglomerans</i>	Unknown	18.37 ± 5.32	44.49 ± 6.55	0 ± 0	33
48D1	<i>Pseudomonas fluorescens</i>	Wisconsin soil	11.81 ± 6.22	62.17 ± 1.59	0 ± 0	0
48G9	<i>Pseudomonas chlororaphis</i>	Wisconsin soil	21.44 ± 3.37	64.28 ± 1.23	1.17 ± 0.22	8
48H11	<i>Pseudomonas brassicacearum</i>	Wisconsin soil	-0.87 ± 0.87	13.14 ± 5.53	0 ± 0	0
88A6	<i>Pseudomonas rhodesiae</i>	Missouri soils	9.34 ± 1.8	31.49 ± 0.6	0 ± 0	6
89F1	<i>Pseudomonas fluorescens</i>	Missouri soils	17.72 ± 4.38	34.18 ± 4.07	0 ± 0	26
90D7A	<i>Pseudomonas fluorescens</i>	Missouri soils	6.31 ± 4.59	15.73 ± 5.47	0 ± 0	10
90F12-1	<i>Pseudomonas rhodesiae</i>	Missouri soils	17.02 ± 1.1	37.39 ± 2.51	0 ± 0	4
90F12-2	<i>Pseudomonas fluorescens</i>	Missouri soils	9.68 ± 6.24	15.05 ± 11.2	0 ± 0	5
93F8	<i>Pseudomonas brassicacearum</i>	Missouri soils	45.99 ± 2.57	72.57 ± 0.42	3.33 ± 0.17	0
94G2	<i>Pseudomonas frederiksbergensis</i>	Missouri soils	12.62 ± 3.21	55.88 ± 0.75	0 ± 0	75
Clinton	<i>Pseudomonas protegens</i>	Ohio soil	20.94 ± 1.64	63.58 ± 1.2	1.33 ± 0.33	0
Darke	<i>Pseudomonas protegens</i>	Ohio soil	28.88 ± 9.12	64.42 ± 1.82	1.67 ± 0.33	7
Delaware	<i>Pseudomonas brassicacearum</i>	Ohio soil	35.02 ± 1.12	62.87 ± 1.52	0.75 ± 0.25	0
Wayne	<i>Pseudomonas protegens</i>	Ohio soil	29.96 ± 6.39	66.24 ± 1.52	2.17 ± 0.44	11
Wood 1	<i>Pseudomonas brassicacearum</i>	Ohio soil	31.49 ± 2.49	68.48 ± 2.01	3.67 ± 0.17	0
Wood 3	<i>Pseudomonas brassicacearum</i>	Ohio soil	27.75 ± 3.42	66.38 ± 1.24	1.25 ± 0.14	1
AP1	<i>Pseudomonas putida</i>	Dr. J. Kloepper	15.19 ± 3.19	34.6 ± 2.95	0 ± 0	0
AP19	<i>Pseudomonas putida</i>	Dr. J. Kloepper	5.91 ± 2.23	30.38 ± 0.73	0 ± 0	8
AP20	<i>Pseudomonas putida</i>	Dr. J. Kloepper	18.14 ± 2.57	41.77 ± 4.07	0 ± 0	0
AP22	<i>Phyllobacterium rubiacearum</i>	Dr. J. Kloepper	0 ± 0	27 ± 1.11	0 ± 0	0
AP24	<i>Phyllobacterium rubiacearum</i>	Dr. J. Kloepper	2.95 ± 2.95	26.16 ± 2.23	0 ± 0	0
AP28	<i>Pseudomonas chlororaphis</i>	Dr. J. Kloepper	59.49 ± 9.5	81.43 ± 3.38	0 ± 0	1
AP35	<i>Ochrobactrum anthropi</i>	Dr. J. Kloepper	9.71 ± 4.98	30.38 ± 5.71	0 ± 0	0
AP36	<i>Ochrobactrum anthropi</i>	Dr. J. Kloepper	0 ± 0	20.25 ± 2.19	0 ± 0	6
AP47 ^h	<i>Rhizobium tropici</i>	Dr. J. Kloepper	0 ± 0	24.47 ± 1.52	0 ± 0	26
AP54 ^h	<i>Pseudomonas protegens</i>	Dr. J. Kloepper	17.72 ± 1.93	48.1 ± 1.93	0 ± 0	39
AP56	<i>Comamonas acidovorans</i>	Dr. J. Kloepper	36.71 ± 3.35	70.46 ± 1.52	4.17 ± 0.83	0
AP58	<i>Comamonas acidovorans</i>	Dr. J. Kloepper	46.41 ± 5.49	75.95 ± 0	5.17 ± 0.67	6
AP59	<i>Comamonas acidovorans</i>	Dr. J. Kloepper	46.83 ± 1.27	73.42 ± 0.73	4.67 ± 0.33	0
AP187	<i>Yersinia frederiksenii</i>	Dr. J. Kloepper	24.47 ± 0.85	58.65 ± 0.42	0 ± 0	18
AP222	<i>Pseudomonas putida</i>	Dr. J. Kloepper	11.39 ± 1.93	26.16 ± 2.57	0 ± 0	0
NA	Cycloheximide solution ⁱ	NA	39.96 ± 0.61	63.01 ± 0.91	3.24 ± 0.31	NA
QST 713	<i>Bacillus subtilis</i> ^j	Cease	NA	NA	NA	12
NA	Negative control ^k	NA	0 ± 0	0 ± 0	0 ± 0	NA

^a All strains except those from Ohio soils and Dr. J. Kloepper were first described by Aly (2009) and Subedi et al. (2019). Strains from Ohio soils were described by McSpadden Gardener et al. (2005).

^b *B. cinerea* mycelial growth and zone of inhibition were recorded in the dual-culture assay for each bacterial treatment replicated three times.

^c The mean percentage growth reduction (GR) of mycelium parallel to bacterial streak (R1) ± standard error from the dual-culture assay.

^d The mean percentage growth reduction of mycelium perpendicular to the bacterial streak (R2) ± standard error from the dual-culture assay.

^e The zone of inhibition (millimeters) distance from the leading edge of the *B. cinerea* mycelium to the edge of the bacterial streak ± standard error from the dual-culture assay.

^f The 60 bacterial strains were evaluated on petunia plants along with positive control (*Bacillus subtilis* QST 713) and negative control (no bacteria). All open flowers were tagged before *B. cinerea* inoculation and rated daily using a disease severity scale (0: no symptoms; 7: senesced because of *B. cinerea*) (Table 2). Each bacterial treatment was replicated 3 times, whereas both controls were replicated 12 times.

^g In the initial greenhouse trial, the score was given to bacterial treatments considering how often each strain appeared in the top 10-ranked bacteria on each rating day. Rank was determined from the fixed effect estimates of treatment effect on rating compared with the negative control (Supplementary Table S1).

^h Identity of strains is based on the results of genome sequencing and percentage taxonomy by CoreBiome 2019.

ⁱ Cycloheximide (58.6 mM in ethanol) was used as the positive control in the dual-culture assay. NA = not applicable.

^j *Bacillus subtilis* QST 713 was used as the positive control in the greenhouse trials.

^k The negative control treatment contained no bacteria.

total number of flowers per plant (Horiuchi and Hori 1980; Strelkov et al. 2006):

$$ID(\%) = \frac{\sum((n_0 \times 0) + (n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3) + (n_4 \times 4) + (n_5 \times 5) + (n_6 \times 6) + (n_7 \times 7))}{N \times 7} \times 100\%$$

Greenhouse validation trial. Experimental design. The seven top-performing strains selected from the initial greenhouse trial were used in the greenhouse validation trial. The same negative (sterile LB medium) and positive (*Bacillus subtilis* QST 713) controls were used in these experiments. The validation trial was conducted twice using a randomized complete block design with 12 replicates of each treatment. Plant material, bacteria treatments, pathogen inoculation, and daily disease severity ratings were the same as described above for the initial greenhouse trial.

Environmental data. Environmental data inside the tents were collected using a weather station (Watchdog Mini Weather Station; Spectrum Technologies, Inc., Plainfield, IL) that took measurements every 30 min. The daily average photosynthetic active radiation (PAR) light (Fig. 2A), relative humidity (Fig. 2B), and temperature (Fig. 2C) were calculated for a 24-h period for each day (days 1 to 12) after *B. cinerea* inoculation. Data were collected every 30 min from time of inoculation (day 0) to project termination (day 12) for daily average PAR light (micromoles per meter² per second) (Fig. 2A), daily average relative humidity (percentage) (Fig. 2B), and daily average temperature (degrees Celsius) (Fig. 2C). Day 0 measurements began at 18:30. All other days are an average of a 24-h period (Fig. 2).

Statistical analysis. Dual-culture assay. All statistical analyses were done in R (R Foundation for Statistical Computing, Vienna, Austria). A principal component analysis using the princomp function (R Core Team 2019) was conducted for the *R1* and *R2* growth measurement values, and a principal component was selected to use as the response variable for analysis of variance. All treatments were compared with the negative control using Dunnett's Comparison.

Initial greenhouse trial. The calculated index of disease was used to select the top-performing bacteria. Square root transformation was used to transform the index of disease. The transformed data were then analyzed with lme4 (Bates et al. 2015) using a fit linear mixed effects model. Fixed effect estimates of treatment effect on rating day

(days 2 to 12) were used to rank the treatments compared with the negative control for each rating day. A scoring system that considered how often each strain appeared in the top 10 ranking for each day was used to select the top-performing strains. If the strain ranked first in day 2, it received 10 points, and if it ranked sixth in day 3, it received 5 points and so on. These points were then added together for all rating days to get the overall disease severity score.

Greenhouse validation trial. The daily ratings collected for the validation trials were used to calculate the area under the disease progress curve (AUDPC) (Shaner and Finney 1977). The square root-transformed AUDPC was analyzed using a fit linear model. The daily ratings were also used to calculate the index of disease, and they were analyzed as described for the initial greenhouse trial.

Results

Dual-culture assay. Twenty of the 60 bacterial strains evaluated in the dual-culture assay resulted in a $\geq 25\%$ *R1* growth reduction (GR) of *B. cinerea*, and 36 strains resulted in a $\geq 50\%$ *R2* growth reduction (Table 1). AP28 resulted in the highest *R1* and *R2* growth reductions at $59.49 \pm 9.50\%$ and $81.43 \pm 3.38\%$, respectively. However, not all strains followed a pattern of having similar *R1* and *R2* percentage growth reductions. For example, 29G9 resulted in one of the highest *R2* growth reductions at $73.69 \pm 2.13\%$ but one of the lowest *R1* growth reductions of $7.35 \pm 7.84\%$. Strain 48H11 resulted in the lowest growth reduction for both *R1* ($-0.87 \pm 0.87\%$) and *R2* ($13.14 \pm 9.58\%$). For *R3* measurements, 35 of the tested strains had no zone of inhibition. Of those strains that did have a zone of inhibition, the largest *R3* value was 5.2 mm for AP58, and the smallest *R3* value was 0.67 mm for 36F3 (Table 1).

The principal component used to compare the treatments with the negative control accounted for 99% of variance. Although 56 strains had a negative estimate, only 35 bacterial strains were statistically different from the negative control based on Dunnett's test at $P < 0.001$ (Fig. 3). Among these were seven strains (AP28, AP59, AP58, 93F8, 14B2, 12H11, and 15G2) that performed better than the positive control. Of these 35 bacteria, only 22 had a zone of inhibition (*R3*) (Table 1). Four strains (48H11, AP22, AP47, and AP36) had positive estimated values, showing that there was no reduced *B. cinerea* mycelial growth compared with the negative control. Eight treatments that resulted in both mycelial growth reduction and a large zone of inhibition were AP59, AP58, 93F8, 14B2, 15H3, AP56, Wood 1, and 36B7 (Table 1).

Initial greenhouse trial for bacterial strain selection. The disease severity index fixed effect estimates of treatment effect on rating day were used to select the top-performing bacteria (Fig. 4). The negative values indicate treatments that performed better than the negative control (less disease), and positive values indicate treatments that performed worse. Looking at 2, 6, and 12 days after the *B. cinerea* inoculation, a majority of the bacterial strains resulted in less gray mold on the plants compared with the negative control. On day 12, 10 bacterial strains performed better than the positive control, *Bacillus subtilis* QST 713 (Fig. 4C).

Fig. 1. Dual-culture assay measurements defined. A dual-culture assay was used to evaluate the collection of 60 bacterial strains. A *Botrytis cinerea* mycelial plug was placed in the center of a petri dish, and each bacterial strain was streaked 2.5 cm away from the plug along both sides. The growth of the mycelium was measured parallel (*R1*) and perpendicular (*R2*) to the bacterial streak. The zone of inhibition (*R3*), which is the distance from the leading edge of the *B. cinerea* mycelium to the edge of the bacterial streak, was also measured.

Table 2. Disease severity rating scale used for *Botrytis cinerea* infection in petunia flowers

Rating	Symptoms
0	No disease
1	≤ 5 small lesions
2	> 5 small lesions
3	Medium-sized lesions
4	Large lesions or medium-sized lesions scattered across the flower
5	Half or more of the flower is covered in lesions
6	The flower is starting to senesce because of <i>B. cinerea</i>
7	The flower is senesced or detached because of <i>B. cinerea</i>

Seven top-performing strains were identified based on an overall disease severity score. The overall disease severity score was calculated using the daily ranking of the strain based on the disease severity index fixed effect estimates of treatment effect on rating day. For example, strain 94G2 received 8 points for day 3, 10 points for day 4, and so on to receive an overall disease severity score of 75 (Table 1 and Supplementary Table S1). The higher score indicated that a treatment resulted in plants with less disease across more days compared with the other treatments (Table 1). Strains AP54, 14B11, and MBSA-3BB1 received overall disease severity scores of 39, 36, and 33, respectively, whereas strains 89F1, 15H3, and AP47 all had scores of 26. The positive control had a score of only 12 (Table 1).

Plants treated individually with the seven top-performing strains had reduced disease severity compared with those treated with the negative control (no bacteria) (Fig. 5). Six days after rating, plants

treated with the top strains (94G2 [20.90 ± 5.36], AP54 [29.45 ± 11.15], 14B11 [22.23 ± 7.94], MBSA-3BB1 [23.98 ± 4.59], 89F1 [44.13 ± 6.51], 15H3 [29.13 ± 5.95], and AP47 [28.20 ± 10.40]) and the positive control (29.47 ± 3.06) had reduced disease severity indices compared with the negative control plants (50.85 ± 4.20). Strain 94G2 resulted in the highest disease reduction, with a 59% decrease in the disease severity index compared with negative control plants, whereas plants treated with 89F1 had a 13% decrease. Negative control plants continued to have a higher disease severity index compared with those treated with either one of the top seven strains or the positive control through the last rating day. On the last day of rating, plants treated with 94G2, AP54, 14B11, MBSA-3BB1, 89F1, 15H3, or AP47 had a lower disease severity index compared with the negative control (78.42 ± 3.42), with indices of 56.51 ± 15.38 , 52.94 ± 10.76 , 38.89 ± 3.97 , 59.05 ± 12.38 , 55.40 ± 0.88 , 42.78 ± 6.16 , and 62.86 ± 3.60 , respectively. Positive control plants were comparable with the bacterial treatments, with a disease severity index of 55.47 ± 3.55 (Fig. 5). Strain 14B11 resulted in the highest disease reduction, with a 50% reduction in the disease severity index compared with negative control plants, whereas plants treated with AP47 had a 19% reduction.

Greenhouse validation trial. All seven strains selected from the initial greenhouse trial reduced disease severity on treated plants in the validation trial. There was a bacterial treatment effect on AUDPC ($P = 0.0013$) (Fig. 6). Dunnett's test was used to determine that 14B11 ($P = 0.0449$), AP54 ($P = 0.0073$), and the positive control ($P = 0.001$) had reduced disease severity over the rating period with AUDPC values of 44.92 ± 2.72 , 42.94 ± 2.43 , and 41.18 ± 2.29 , respectively (Fig. 6). The negative control had an AUDPC of 51.07 ± 1.91 . Strains 89F1, 94G2, 15H3, AP47, and MBSA-3BB1 had lower but not significantly different AUDPC values of 45.59 ± 2.16 ($P = 0.107$), 46.57 ± 2.30 ($P = 0.201$), 48.65 ± 1.86 ($P = 0.571$), 49.49 ± 1.67 ($P = 0.718$), and 49.98 ± 2.47 ($P = 0.720$), respectively, when compared with the negative control (Fig. 6).

The disease severity index fixed effect estimates of treatment effect on rating day were also used to identify the top-performing strains among the seven tested (Fig. 7). Strain 89F1 reduced disease severity more than the positive control on day 2 (Fig. 7A), and then, it remained in the top four strains for disease reduction through day 12 (Fig. 7C). Strain AP54 was one of the top two strains on each rating day. Strain 14B11 appeared in the top three strains on day 2 (Fig. 7A) and remained in the top three strains through the last day of rating. Strain MBSA-3BB1 had a higher disease severity index than the negative control on day 2, but then, it had a reduced disease severity index compared with the negative control by day 6. Strains 15H3 and AP47 had reduced disease severity compared with the negative control each rating day. In the estimate rankings, 94G2 was among the top three strains on day 12 (Fig. 7C).

The three top-performing strains selected through calculating the AUDPC and daily disease severity index were AP54, 14B11, and 89F1. Plants treated with AP54 or 14B11 had a reduced AUDPC value (Fig. 6) and disease severity index (Fig. 7). Although plants treated with 89F1 did not have a significant decrease in AUDPC values, there was a consistent reduction in the disease severity index compared with the negative control.

These three strains had a decreased disease severity index across all rating days. Considering the two validation experiments, the extent of the reduction in disease severity varied because of the recorded environmental conditions (Fig. 2). Compared with experiment 1, the average relative humidity was higher and the average temperature was lower in experiment 2, which are conditions more conducive to *B. cinerea* infection. This environmental effect is reflected in the disease severity index. For example, the negative control in experiment 1 on day 2 had a disease severity index of 20.39 ± 1.75 , and the positive control had a disease severity index of 13.32 ± 1.81 (Fig. 8A). In experiment 2 on day 2, the negative control had a disease severity index of 31.31 ± 3.57 , whereas the positive control had a disease severity index of 21.66 ± 2.67 (Fig. 8B). In experiment 1, there was a clear separation of the treatments by day 7, where the top three strains resulted in disease severity indices in a range

Fig. 2. Environmental data for the greenhouse validation trial. Solid lines and black circles represent experiment 1, and dashed lines and white circles represent experiment 2. Data were collected every 30 min from time of inoculation (day 0) to project termination (day 12) for **A**, daily average photosynthetic active radiation (PAR) light (micromoles per meter² per second); **B**, daily average relative humidity (percentage); and **C**, daily average temperature (degrees Celsius). Day 0 measurements began at 18:30. All other days are an average of a 24-h period.

between the negative (72.96 ± 4.65) and positive (54.45 ± 4.34) controls (Fig. 8A). Disease progressed faster in experiment 2 because clear separation in the treatments was observed by day 3, where the top three strains resulted in disease severity indices in a range between the negative (46.50 ± 4.43) and positive (34.41 ± 3.33) controls. By day 12 in experiment 1, the positive control plants had a 27% reduction in the disease severity index compared with the negative control, whereas the disease severity indices were reduced by 18, 19, and 14% for AP54, 14B11, and 89F1, respectively. By day 12 in experiment 2, positive control plants had only a 7% reduction in disease severity index compared with the negative control, whereas there were reductions of 10, 2, and 1% for AP54, 14B11, and 89F1, respectively (Fig. 8).

Discussion

Biocontrol products give growers additional tools to manage pathogens, and biocontrol options are especially needed for *B. cinerea* because it is becoming increasingly resistant to chemical fungicides (Muñoz et al. 2019). Beneficial bacteria have previously shown potential as biocontrol agents in the management of gray mold (Barka et al. 2002; Yildiz et al. 2007), specifically in greenhouse floriculture crops (Gould et al. 1996; Redmond et al. 1987). This study aimed to identify potential biocontrol agents from a collection of 60 bacterial strains for the control of gray mold in greenhouse floriculture crops. The original bacterial collection was evaluated in a dual-culture assay, where 35 strains were selected as top performers for the reduction of *B. cinerea* in vitro along with an initial greenhouse trial where seven strains were selected as top performers for the reduction of gray mold severity in planta. The seven top-performing strains from the initial greenhouse trial were then evaluated in the greenhouse validation trial, and three top-performing strains (*Pseudomonas fluorescens*

89F1, *Pseudomonas chlororaphis* 14B11, and *Pseudomonas protegens* AP54) were selected.

In vitro inhibition assays are often used as an initial method to narrow down a collection of potential antagonists (Kefi et al. 2015) or to further characterize a collection (Trotel-Aziz et al. 2008). In this study, 60 previously identified beneficial bacteria were characterized for the ability to reduce *B. cinerea* growth in a dual-culture assay, but this was not used as the sole method for selecting top-performing bacteria. The mycelial growth (*R1* and *R2*) reduction and zone of inhibition can be attributed to antifungal compound production by antagonists, such as antibiotics (like 2, 4-diacetophloroglucinol), enzymes (like chitinase), or volatiles (like hydrogen cyanide), that can inhibit growth of a plant pathogen (Kerr 1999). Specifically, a zone of inhibition is a good indicator of antifungal compound production (Chand and Logan 1984; Köhl et al. 2019; Royse and Ries 1978).

Twenty-five strains had zones of inhibition, whereas a few strains, like *Pseudomonas protegens* AP58, had both a large mycelial growth reduction (*R1* and *R2*) and a large zone of inhibition, indicating the potential for antifungal compound production. A reduction in mycelial growth with no zone of inhibition is an indication of varying growth rates between the antagonist and the pathogen (Fokkema 1973). *Pseudomonas chlororaphis* AP28 had the largest mycelial growth reduction (*R1* and *R2*) with no zone of inhibition. Wang et al. (2009) evaluated 238 bacterial isolates in an in vitro assay against *B. cinerea* and used the zone of inhibition and rate of inhibition (based on *R1* and *R2*) to select three bacteria to be further evaluated. These three strains were evaluated on detached tomato leaves, and one *Bacillus subtilis* strain was identified based on the reduction in lesion diameter on leaves treated with the strain compared with the untreated control. Using a similar selection criterion in our study, only 8 strains of 60 had both a zone of inhibition and reduced

Fig. 3. Estimates from selected principal components (PCs) compared with the negative control. A PC analysis was conducted with the two *Botrytis cinerea* growth measurements from the dual-culture assay, growth of the mycelium measured parallel (*R1*) and perpendicular (*R2*) to the bacterial streak. The selected PC was used to do a Dunnett's Comparison of the 60 bacterial strains and the positive control (cycloheximide solution) with the negative control (no bacteria). Treatments that reduced the *B. cinerea* growth compared with the negative control have a negative value, whereas those that did not have a positive value. Black bars are different than the negative control with $P < 0.001$, and the white bar with slanted lines represents the positive control. Those treatments not different from the negative control ($P > 0.001$) are gray. The error bars denote standard error.

mycelial growth (Table 1). Careful conclusions must be drawn from the dual-culture assay because it has been shown that growth media type can affect the antagonist's antibiotic production (Whipps 1987). Also, this zone of inhibition could be a result of metabolite production by the pathogen, which is induced by the antagonist instead of from the antagonist itself (Fokkema 1973).

The full collection of bacteria was also evaluated in the initial greenhouse trial for the strains' ability to reduce *B. cinerea* infection in a greenhouse production setting, which accounts for the plant, microbe, and environment interactions needed for successful biocontrol efficacy (Pal and McSpadden Gardener 2006). Flowers were evaluated daily for disease severity because they are more susceptible to

Fig. 4. Initial greenhouse trial disease severity index treatment effects compared with the negative control. Treatment effects compared with the negative control for **A**, day 2; **B**, day 6; and **C**, day 12 were extracted from the linear mixed effects model used with the square root-transformed disease severity index data. The 60 bacterial strains and positive control (*Bacillus subtilis* QST 713) were compared with the negative control (no bacteria). Negative values indicate the treatments that resulted in reduced disease severity compared with the negative control, whereas the positive values mean that the plants had a higher disease severity. The positive control is represented by the white bars with slanted lines. Each treatment was replicated three times, whereas the controls were replicated 12 times each.

B. cinerea infection than vegetative tissues (Dik and Wubben 2007). Seven bacteria were identified that reduced gray mold severity on petunia flowers. Plants treated with the top seven bacterial strains had consistently reduced disease severity compared with the negative control plants. Some bacterial treatments also performed as well or better than the commercial biopesticide used as the positive control (*Bacillus subtilis* QST 713). The reduction in gray mold on flowers after application of beneficial organisms supports what has been reported in previous studies with cut roses sprayed with yeast or bacteria (Redmond et al. 1987) and petunia flowers sprayed with bacteria (Gould et al. 1996).

In our study, the dual-culture assay did not predict the top-performing strains identified through the initial greenhouse trial.

Fig. 5. Initial greenhouse trial disease severity index across all rating days of the seven top-performing strains. The disease severity index was calculated using the daily ratings of individual flowers. Plants were rated daily for 11 days. The negative control (no bacteria) is represented with the black upright triangle, and the positive control (*Bacillus subtilis* QST 713) is represented by the white diamond. Each bacterial treatment was replicated three times, whereas the controls were replicated 12 times each. The error bars denote standard error.

Fig. 6. Area under the disease progress curve (AUDPC) calculated from severity ratings collected during the greenhouse validation trial. Treatment effects compared with the negative control extracted from the linear mixed effects model used with the square root-transformed AUDPC data. The positive control (*Bacillus subtilis* QST 713) is represented by the white bar with slanted lines. The negative control (no bacteria) is represented by the black bar. Each treatment was replicated 12 times. Error bars show standard error, and asterisks indicate significance. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Pantoea agglomerans MBSA-3BB1, *Pseudomonas protegens* AP54, *Pseudomonas fluorescens* 89F1, *Pseudomonas frederiksbergensis* 94G2, and *Rhizobium tropici* AP47 did not have a zone of

Fig. 7. Disease severity index treatment effects compared with the negative control for greenhouse validation trial. Treatment effects compared with the negative control for **A**, day 2; **B**, day 6; and **C**, day 12 were extracted from the linear mixed effects model used with the square root-transformed disease severity index data. The seven bacterial strains and positive control (*Bacillus subtilis* QST 713) were compared with the negative control (no bacteria). Negative values indicate the treatments that resulted in reduced disease severity compared with the negative control, whereas the positive values mean that the plants had a higher disease severity. The positive control is represented by the white bars with slanted lines. Each treatment was replicated 12 times.

inhibition in the dual-culture assay. Only *Pseudomonas chlororaphis* 14B11 and *Pseudomonas protegens* 15H3 had a zone of inhibition. *Pseudomonas protegens* 15H3 was the only one of the top seven strains that was selected as a top performer in the dual-culture assay for both zone of inhibition and reduction of mycelial growth. Similarly, in a study with wheat, selected beneficial bacteria from a growth chamber assay were evaluated for the control of *Pythium* spp. through both an in vitro assay and a field study. They found that, although the top-performing strains from the field study showed growth reduction of most *Pythium* spp. tested, the best-performing bacteria in the in vitro assay were not the most effective in the field (Milus and Rothrock 1997).

Using in planta studies as a screening method is challenging in some cases because of the size of the bacterial collection of interest. Gould et al. (1996) developed a petal tissue bioassay to evaluate 172 bacterial isolates for the control of *B. cinerea*, which allowed for more rapid screening of the collection. However, in vitro assays are not always an option for investigating plant-microbe interactions. When selecting bacteria for the biocontrol of *Pseudomonas syringae* pv. *tomato* from a collection of approximately 70 strains, greenhouse trials were used as an alternative to an in vitro study to identify a few strains for field testing. Two strains were identified for their ability to reduce disease through this selection process (Wilson et al. 2002). In our study, most of the top-performing bacteria would have been overlooked if the dual-culture assay was the sole means of selecting strains for greenhouse validation. A dual-culture assay can lead to a narrow view of potential biocontrol candidates because it focuses on one or few modes of action. A screening assay that considers the complexity of the system, including the pathogen, antagonist,

plant, and environment, is needed to capture potential biocontrol candidates (Köhl et al. 2019). An augmented design was used in our study to screen the entire bacterial collection in the initial greenhouse trial and select top strains for further evaluation. This type of experimental design is used in plant breeding programs to allow for low replication of varieties, whereas the checks or controls are overreplicated (Federer and Raghavarao 1975).

Seven strains selected from the initial greenhouse trial were evaluated in the validation trial to confirm the ability of these strains to reduce gray mold severity in petunia. Although all seven strains performed better than the negative control, three strains were identified as the top performers based on AUDPC and disease severity index. *Pseudomonas protegens* AP54 and *Pseudomonas chlororaphis* 14B11 were top performers in AUDPC and across all rating days for disease severity index, whereas *Pseudomonas fluorescens* 89F1 was a top performer when looking at reduced disease severity index. These three top strains were from the genus *Pseudomonas*. *Pseudomonas* spp. have shown potential for biocontrol of *B. cinerea* infection in various crops. Several *Pseudomonas fluorescens* strains were identified based on their ability to reduce disease severity caused by *B. cinerea* in greenhouse-grown tomato plants, with some of the strains capable of reducing disease when used in combination with fungicides (Yildiz et al. 2007). *Pseudomonas* sp. PsJN isolated from onion root was used to successfully reduce *B. cinerea* infection in grapevine plantlets (Barka et al. 2002), and a *Pseudomonas fluorescens* strain, originally isolated from petunia leaves and flowers, reduced *B. cinerea* infection on petunia flowers (Gould et al. 1996).

Conclusion. The greenhouse trials and rating methods that focused on flower disease severity were successful in identifying potential biocontrol agents for gray mold in petunia. These selected beneficial bacterial strains have the potential to be formulated into biocontrol products for the ornamental greenhouse industry. Biocontrol products added into an integrated pest management plan can help the ornamental greenhouse industry move toward a more sustainable production system. Future studies will need to identify the modes of action for disease control so that the bacteria can be utilized in the most effective way. Understanding more about the mode of action will also provide information for the best method to formulate and implement these selected bacteria for the control of *B. cinerea* in greenhouse floriculture production.

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Fig. 8. Disease severity index across all rating days of the three top-performing strains from the greenhouse validation trial. The disease severity index was calculated using the daily ratings of individual flowers. Plants were rated daily for 11 days for both time replicates in A, experiment 1 and B, experiment 2. The negative control (no bacteria) is represented by the black squares, and the positive control (*Bacillus subtilis* QST 713) is represented by the white triangles. Each treatment was replicated 12 times. The error bars denote standard error.

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