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Margaret T. Mmbaga
Tennessee State University

Lucas M. Mackasmiel
Tennessee State University

Frank A. Mrema
Alcorn State University

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Evaluation of Biological Agents for Control of *Macrophomina* Root Rot and Powdery Mildew in Flowering Dogwood (*Cornus florida* L.)

Margaret T. Mmbaga¹ and Lucas M. Mackasmiel

Department of Agricultural and Environmental Sciences, College of Agriculture, Tennessee State University, Nashville, TN 37209

Frank A. Mrema

Research and Applied Sciences, Alcorn State University, Lorman, MS 39096

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Abstract. Six biological control agents (BCAs) (two bacteria, two fungi, and two yeasts) that were previously shown to be effective against powdery mildew (*Erysiphe pulchra*) were tested for efficacy against *Macrophomina phaseolina* root rot on flowering dogwood (*Cornus florida*) in the greenhouse. Two of the bacterial isolates, *Stenotrophomonas* sp. (B17A) and *Serratia* sp. (B17B), were effective in controlling both *macrophomina* root rot and powdery mildew, similar to fungicide control thiophanate methyl, when roots were drenched with the six BCAs individually. In addition, the two bacterial BCAs improved plant growth with respect to stem diameter, stem length, dry weight, and green foliage compared with fungicide-treated plants or nontreated controls grown in sterile soil. These results confirm previous results in which B17A and B17B suppressed powdery mildew and also promoted plant growth in flowering dogwood. Although *macrophomina* root rot has been previously reported as a potential problem in flowering dogwood, especially in field conditions, simultaneous infection with *macrophomina* root rot and powdery mildew has not been previously reported. This study confirmed that *M. phaseolina* infection was characterized by stubby roots and black root lesions, and plants infected with both powdery mildew and *macrophomina* root rot had smaller root mass compared with fungicide-treated plants. Neither of the two pathogens killed their host plants, but compounded infections significantly reduced the plant root system and plant growth. The efficacy of the two bacterial isolates in controlling both powdery mildew and *macrophomina* root rot suggests their potential utilization in controlling both diseases in dogwood nursery production and in other plants that are hosts to both powdery mildew and *macrophomina* root rot. Plant growth promoted by the two BCAs may be attributed to powdery mildew and *macrophomina* root rot control, but comparisons between fungicide-treated plants and control plants not inoculated with BCAs or root rot pathogen suggested that the two BCAs may play a role as bio-stimulants in growth enhancement. These results also suggest that the two biocontrol agents are not phytotoxic to dogwood.

Macrophomina phaseolina is a nonspecialized soil-borne pathogen that can become a problem by causing root rot, charcoal rot, collar rot, damping-off, wilt, leaf blight, and stem blight in both agricultural and natural or landscape environments. More than 500 plant species are affected across ≈100 families, including the dogwood family (Farr et al., 1989; Smith and Carvil, 1977). Woody ornamental plants affected by this fungus include pine, douglas fir, and the highly valued

ornamental flowering dogwood (*Cornus florida*) (Barnard and Gilly, 1986; Hodges, 1962; Mmbaga et al., 2018; Rowan, 1971; Seymour, 1969a, 1969b; Smith and Bega, 1964). *Macrophomina phaseolina* effects on woody hosts are primarily on seedlings and young plants, causing severe damping-off, especially in stressful environments. Recently *M. phaseolina* was reported to cause cankers in seedlings of flowering dogwood (Mmbaga et al., 2018). The greatest economic impact of *M. phaseolina* is on agronomic crops, such as soybean, corn, sorghum, sunflower, and cotton, where it is associated with severe crop losses (Khan, 2007; Lotfalinezhad et al., 2013; Su et al., 2000; Wrather, 1995; Wyllie, 1988). Although initial isolation of *M. phaseolina* from dogwood was from plants that were also infected with *Erysiphe pulchra* (Mmbaga et al., 2018), the impact of compounded infections on plant growth has not been evaluated. Such

information would have significant implications on disease management in nursery production systems that have been already devastated by powdery mildew since the disease emerged in early 1990s (Mmbaga, 2000; Windham, 1994).

The main symptoms of powdery mildew are a powdery appearance on plant foliage, stunted plant growth, defoliation, and plant health decline (Chartfield and Rose, 1996; Mmbaga et al., 2007). Leaf scorching and leaf reddening has also been associated with dogwood powdery mildew (Mmbaga, 2000; Mmbaga and Sauv , 2004a; Mmbaga et al., 2004; Windham, 1994). Studies on powdery mildew in oak (*Quercus robur*) leaves reported increases in plant respiration and transpiration and reduced leaf life-span that may lead to decreased carbon uptake over the growth season (Hajji et al., 2009). A similar phenomenon may occur in dogwoods infected with powdery mildew. Recent studies have shown that *M. phaseolina* causes stubby roots and reduces root mass in seedlings of flowering dogwoods (Mmbaga et al., 2018). This study was conducted to evaluate the impact of compounded infection from *M. phaseolina* and *E. pulchra* on dogwood plant growth; such information would provide a better understanding of factors that may contribute to dogwood decline.

Fungicides are routinely used to control powdery mildew in nursery production of flowering dogwood (Hagan et al., 2005; Li et al., 2009; Mmbaga and Sauv , 2004b; Windham, 1994), but that practice has caused concerns over environmental and health hazards to human applicators and nontarget organisms including beneficial microflora. Previous studies have shown that native plants in natural environments, where fungicides have never been used, harbor microorganisms that suppress powdery mildew in flowering dogwood (Mmbaga and Sauv , 2009; Mmbaga et al., 2007, 2016). Out of the microorganisms isolated from native plants in natural environments, *Stenotrophomonas* sp. (B17A) and *Serratia* sp. (B17B) were highly effective in controlling powdery mildew in seedling populations by disrupting spore germination (Mmbaga et al., 2016). In addition, the applications of the two bacteria by root drenching suppressed powdery mildew similarly to foliage sprays and suggested that the BCAs may also cause induced systemic resistance (ISR), which may suppress other fungal pathogens including root rot pathogens (Mmbaga et al., 2016). The objectives of this study were to 1) evaluate the efficiency of six previously selected microbial isolates (B17A, B17B, F13, F16, Y4, and Y14) as biological control agents for *macrophomina* root rot and powdery mildew in *C. florida*; and 2) determine the effect of the BCAs on plant health and plant growth.

Materials and Methods

Plant material, M. phaseolina inoculum preparation, and plant inoculation. Dogwood

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¹Corresponding author. E-mail: mmmmbaga@tnstate.edu.

seeds for this study were collected from field-grown *C. florida* at Otis Floyd Nursery Research Center experimental farm (Tennessee State University) in McMinnville, TN and vernalized at 4 °C to break dormancy. *M. phaseolina* inoculum, previously isolated from dogwood (Mmbaga et al., 2018), was prepared from 5 d-old cultures grown on potato dextrose agar (PDA). Mycelia was scraped from the PDA into sterilized water and macerated using a sterilized hand-held blender. Mycelial fragments were counted using a haemocytometer and used as propagules adjusted to a concentration of 5×10^4 propagules/mL. The inoculum suspension was applied to heat-sterilized media consisting of Morton's Grow Mix #2 (Morton's Horticultural Supplies Inc., McMinnville, TN). Thirty mL of the inoculum suspension was applied in 10.2 cm plastic pots filled with heat-sterilized media, mixed thoroughly, and allowed to colonize the media for seven days before planting. Dogwood seedlings were then planted in the *M. phaseolina*-infested media and control plants were planted in heat-sterilized media. All plants were maintained in a greenhouse at 28 ± 3 °C, and watered daily by drip irrigation.

BCA inoculum preparation and plant treatment with the BCAs and fungicide. Experimental treatments consisted of six BCAs including two bacteria, *Stenotrophomonas* sp. (B17A) and *Serratia* sp. (B17B), previously selected for dogwood powdery mildew control (Mmbaga et al., 2016), two fungi, *Acremonium alternatum* (F16) and *Penicillium* spp. (F13), and two yeasts, unidentified species (Y4) and *Rhodospiridium* sp. (Y14), previously selected as potential BCAs for dogwood powdery mildew (Mmbaga et al., 2007). Bacterial BCA inocula were prepared from 24 h-old cultures grown on nutrient broth (NB) containing 1.0 g meat extract; 1.0 g yeast extract; 5.0 g peptone; and 5.0 g sodium chloride per L. After 24 h growth in NB, cells

were pelleted by centrifugation, washed twice in sterile water, and then re-suspended in sterile water containing 0.05% Tween 20. The BCAs were then grown in nutrient agar and inocula of 3×10^6 colony forming units (cfu) per mL was prepared from 24 to 48 h cultures. Yeast inocula were prepared from 7 d-old cultures grown on potato dextrose agar (PDA) and a concentration of 3×10^6 cfu per mL was used. Fungal BCA inocula was prepared from 7 d-old cultures grown on PDA and adjusted to 4×10^6 spores/mL, counted using a haemocytometer. Inocula concentrations of bacteria and yeast BCAs were estimated using optical density readings using a graph curve, previously developed to correspond with specified cfu of each BCA.

The application of BCAs was by drenching to treat the roots using 20 mL of BCA inoculum suspension applied to each 10.2 cm planting container starting in early June/late May, when powdery mildew symptoms were first observed. Efficacy of the six BCAs was compared with the fungicide thiophanate-methyl (Cleary's 3336TM F; Cleary Chemical Corp., Dayton, NJ) and two controls, one consisting of *M. phaseolina* with no BCAs and another control in sterile soil with no BCA and no *M. phaseolina*. Powdery mildew inoculum was from natural airborne spores from previously infected plants placed randomly in the greenhouse. Application of BCAs was repeated at 7 to 10 d intervals through early September/late August to coincide with powdery mildew treatments. The fungicide was prepared at a concentration of 1 mL per 727.3 mL (v/v) water, equivalent to 18 fl oz/100 gallons according to the manufacturer's recommendations (Cleary's Chemicals Corporation, Dayton, NJ). Fungicide application was by root drenching in which media was soaked through root zone using 20 mL per container every 14 d. Each treatment was replicated and comprised four containers with three plants per container.

Treatments were arranged in a randomized complete block design.

Assessment of diseases. Powdery mildew severity was evaluated monthly on a scale of 0 to 5 in which 0 = no infection; 1 = 1% to 10%; 2 = 11% to 25%; 3 = 26% to 50%; 4 = 51% to 75%; and 5 = 76% to 100% of the plant foliage covered with powdery mildew symptoms starting one month after planting. The experiment was ended 90 d after plant inoculation, roots were gently cleaned to remove soil particles, and macrophomina root rot disease severity was evaluated based on root discolorations and deformities, such as root stubbiness and presence or absence of small feeder roots, and visual root mass. Root rot incidence was estimated on a scale of 0 to 5 in which 0 = no infection; 1 = 1% to 10%; 2 = 11% to 25%; 3 = 26% to 50%; 4 = 51% to 75%; and 5 = 76% to 100% of roots displaying root lesions. Re-isolation of *M. phaseolina* was done on PDA to confirm the presence of *M. phaseolina* in the root lesions. Some root samples were cleared using 1% KOH for 24 h and stained with 0.05% aniline blue for observation of microsclerotia under a compound microscope.

Effect of the biocontrol isolates on plant growth. Plant growth was evaluated based on stem length, stem diameter at ≈ 6 cm above soil level, visual root mass, and oven dry weight based on drying to constant weight. Data were analyzed using SAS 9.1 (SAS Institute, Inc., Cary, NC) general linear models procedure and Fisher's least significant difference test at $P < 0.05$.

Results and Discussion

Plant infection with *M. phaseolina* and *E. pulchra*. Dogwood seedlings infected with *M. phaseolina* and *E. pulchra* exhibited significant visual differences in plant color and plant size (Fig. 1). Plants treated with bacterial agents B17A and B17B grew consistently

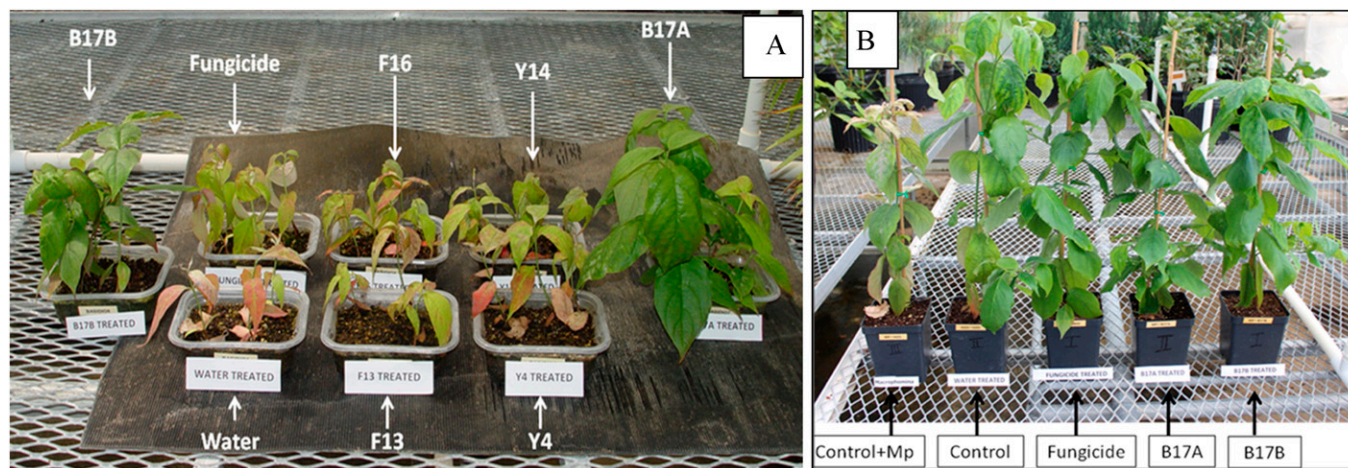


Fig. 1. (A) Effect of six biocontrol agents (BCA) (B17A, B17B, F13, F16, Y4, and Y14), compared with the fungicide thiophanate methyl (fungicide) and nontreated control (water) on dogwood (*Cornus florida*) seedlings planted in heat sterilized soil infested with *Macrophomina phaseolina* and then exposed to airborne spores of *Erysiphe pulchra*, with treatment applied by soil drenching. (B) Overall plant growth and powdery mildew severity in plants treated with bacterial BCA (B17A and B17B) and fungicide (fungicide) compared with control plants with macrophomina root rot and no BCA (Control+Mp) and control plants grown in sterile soil with no BCAs and no macrophomina root rot (Control).

larger and greener than those treated with thiophanate methyl, or with fungal agents (F13 and F16) or yeast agents (Y4 and Y14). Control plants grown in macrophomina-infested media with no BCA (Control+Mp) had greater powdery mildew severity than control plants grown with no *Macrophomina*

and no BCA (Figs. 1B and 2). Although the differences were significant in only Expt. 1, they suggest that plants infected with *Macrophomina* root rot are likely to have higher powdery mildew severity. Some reddish coloration of leaves was observed on all treatments except plants treated with B17A and

B17B (Fig. 1A). The reddish color on leaves obscured the evaluation of powdery appearance associated with powdery mildew disease. However, reddish coloration of leaves has been previously associated with powdery mildew symptoms (Windham, 1994), and it was assumed that the more intense reddish color on leaves was associated with higher incidence of powdery mildew (Fig. 1A). Plants inoculated with *M. phaseolina* as nontreated controls with no BCA and plants treated with yeast and fungal BCAs (Y14, Y4, F13, and F16) displayed root discolorations and black lesions, and stubby roots with few feeder roots (Fig. 3A–C). These observations suggested that the yeast and fungal BCAs were not effective in reducing root rot (data not shown). Microscopic observations revealed many microsclerotia resting structures in inoculated and not in noninoculated plants. *M. phaseolina* was re-isolated from root lesions and thus confirmed the presence of *M. phaseolina* as a causal agent for the root rot.

Effect of the biocontrol agents on disease severity. Plants grown in macrophomina-infested media without any disease control developed the highest powdery mildew severity and exhibited early defoliation compared with those treated with effective BCAs or fungicide (Fig. 1). Differences in powdery mildew severity between BCA treatments

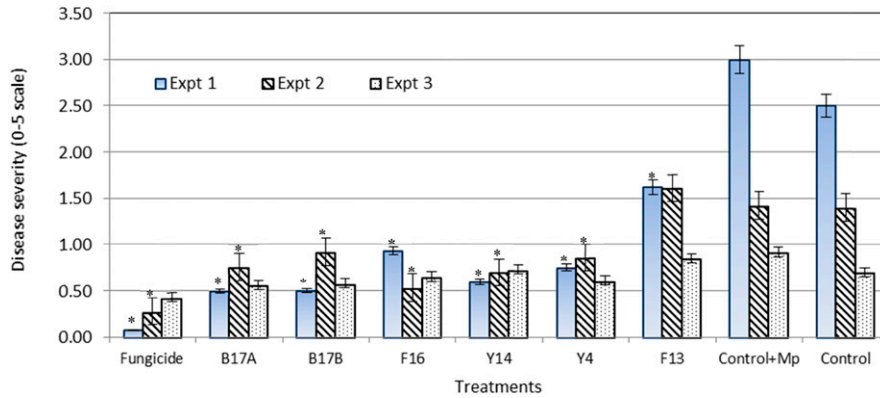


Fig. 2. Powdery mildew disease severity on *Cornus florida* plants grown in soil infested with *Macrophomina phaseolina* (Mp) and drenched with six biocontrol agents (B17A, B17B, F16, Y14, Y4, and F13), thiophanate methyl, and nontreated control (Control+Mp) and nontreated control with no macrophomina and no biocontrol (control). Disease severity on a 0 to 5 scale in which 0 = no infection; 1 = 1% to 10%; 2 = 11% to 25%; 3 = 26% to 50%; 4 = 51% to 75%; and 5 = 76% to 100% of plant covered with powdery mildew symptoms. *Significantly different from nontreated control (Control) and Control+Mp at $P < 0.05$ in each experiment. Standard error bars at $P < 0.05$

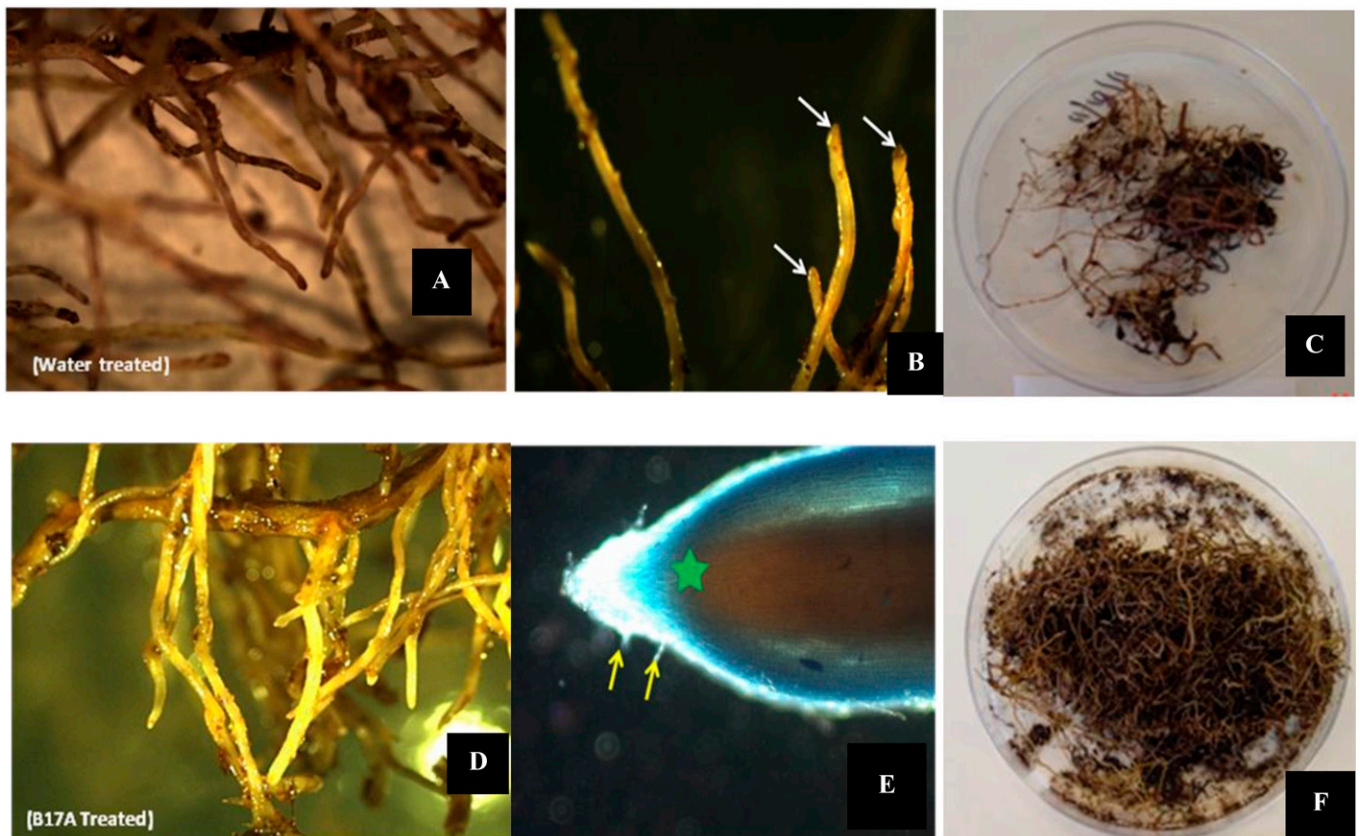


Fig. 3. Roots of flowering dogwood seedlings grown in soil infested with *Macrophomina phaseolina* and drenched with selected biocontrol agents and the fungicide thiophanate methyl in the greenhouse: (A) root discolorations and (B and C) stubby roots with no feeder roots compared with (D) healthy roots with plenty of feeder roots in effective treatments (B17A, B17B, Fungicide, and control with no macrophomina), (E) root hairs (arrows), and (F) larger root mass in plants treated with effective biocontrol agents B17A, B17B, and fungicide.

were significant at $P = 0.01$ in Expts. 1 and 2 but not significant in Expt. 3 (Fig. 2). Treatments with fungicide had the lowest powdery mildew disease incidence in all three experiments. Treatments with bacterial BCAs (B17A and B17B) and treatment with fungal and yeast BCAs (F16, Y14, and Y4) were also highly effective in suppressing powdery mildew in Expts. 1 and 2, but fungal isolate F13 was not effective (Fig. 2). In Expt. 1, control treatments with *M. phaseolina* and no BCA treatments had the highest powdery mildew severity followed by the control with no *M. phaseolina* and no BCA treatment, while in Expt. 2, the two control treatments and fungal BCA F13 had the highest powdery mildew severity compared with other treatments, the differences being significant at $P = 0.05$ (Fig. 2). Overall, powdery mildew severity was very low in Expt. 3 and there were no significant differences in powdery mildew severity between treatments (Fig. 2). Variations in powdery mildew severity in different years are not uncommon because any variations in the amount of airborne inoculum or temperature and moisture impact disease severity (Mmbaga, 2000, 2002).

Results from this study confirmed previous results showing that B17A and B17B were effective biocontrol agents that may be used to reduce the use of conventional fungicide treatment (Mmbaga et al., 2016). Additional biocontrol agents Y14, Y4, and F16 were confirmed as effective against powdery mildew, and may also be used to reduce conventional fungicide use in powdery mildew control (Fig. 2). However, Y14, Y4, and F16 were not effective in controlling macrophomina root rot (as explained above). Plants treated with bacterial isolates B17A, B17B, or fungicide exhibited healthy roots with plenty of small feeder roots similar to control plants grown in sterile soil with no *M. phaseolina* (Fig. 3D–F); microscopic observation of roots from B17A, B17B, and fungicide treatments revealed abundant root hairs (Fig. 3E). These results showed that B17A and B17B were effective in controlling both powdery mildew and macrophomina root rot.

A number of biocontrol agents have been tested for the control of *M. phaseolina* in food and cash crops in many parts of the world, and *Trichoderma viride*, *T. harzianum*, *Pseudomonas fluorescens*, and *Bacillus subtilis* have been shown to inhibit *M. phaseolina* in culture and in the greenhouse environment (Kumar, 2013). The efficacy of the two bacterial isolates in our study in controlling both powdery mildew and macrophomina root rot suggests their potential utilization in dogwood nursery production and in other plants that are hosts to both powdery mildew and macrophomina root rot.

Effect of the biocontrol isolates on plant growth. Significant differences in plant growth were observed between treatments, as shown for stem length, stem diameter as well as oven dry weight ($P < 0.01$; Figs. 1, 4, and 5). Plants treated with bacterial BCAs grew significantly larger than fungicide-treated plants and their leaves maintained their green color for longer

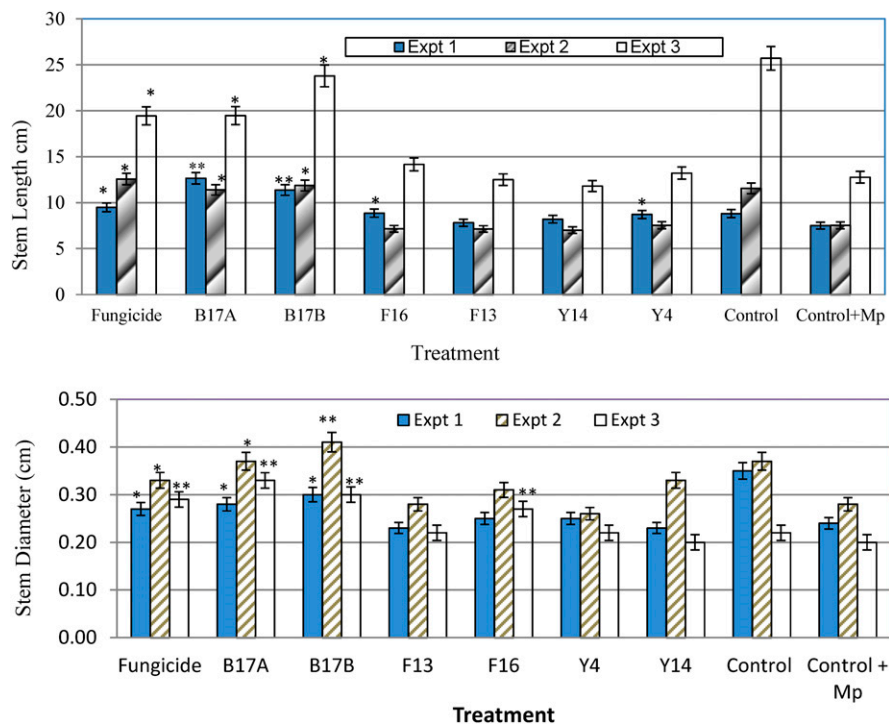


Fig. 4. Stem height and stem diameter as a measure of plant growth in *Cornus florida* seedlings grown in soil infested with *Macrophomina phaseolina* and drenched with biocontrol agents (B17A, B17B, F13, F16, Y4, and Y14) and fungicide thiophanate methyl (Fungicide), compared with nontreated control with macrophomina (Control+Mp) and plants grown in sterile soil with no *M. phaseolina* (Control). *Significantly different from nontreated control with macrophomina (Control+Mp). **Significantly different from Control and Control+Mp at $P < 0.05$ in each experiment. Standard error bars at $P < 0.05$

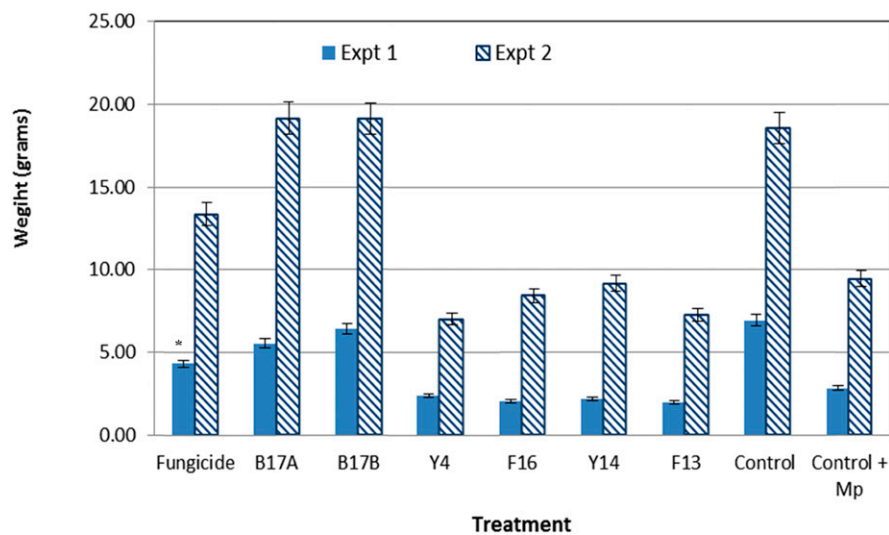


Fig. 5. Oven dry weight of *Cornus florida* seedlings grown in soil infested with *Macrophomina phaseolina* and treated with biocontrol agents (B17A, B17B, F13, F16, Y4, and Y14) and compared with the fungicide thiophanate methyl (Fungicide), nontreated control (Control+Mp), and plants grown in sterile soil with no macrophomina (Control). No data were taken in Expt 1. Standard error bars at $P < 0.05$.

periods up to the end of the growing season compared with all other treatments with *M. phaseolina*. Plants treated with the two bacterial BCA agents, B17A and B17B, developed more branching, larger leaves and tall stems, even in the presence of *M. phaseolina*, compared with fungicide-treated plants and other BCA treatments (Figs. 1, 4, and 5). Although

the fungicide was slightly more effective than the BCAs (B17A and B17B) in powdery mildew control (Fig. 2), the BCAs promoted stem diameter and oven dry weight better than the fungicide treatment and the macrophomina infected control with no BCA (Fig. 4). Plants exhibited larger stem height and dry weight in Expt. 3 compared with Expts. 1 and 2 (Figs. 4

and 5). The difference may be explained by unintentional use of slightly larger plants in Expt. 3 compared with Expts. 1 and 2. However, no big differences were observed on stem diameters.

The greatest stem height in Expts. 1 and 2 was with treatments with B17A and B17B followed by fungicide, while in experiment 3 control treatment with no *M. phaseolina* or BCA and B17B had the greatest stem height followed by B17A and fungicide (Fig. 4). Similarly, control treatment in which plants were grown in sterilized media with no *M. phaseolina* or BCA had the greatest stem diameter in Expt. 1 followed by B17B and B17A and fungicide suggesting, that macrophomina root rot had an effect on plant growth and that B17B and B17A positively impacted plant growth by controlling macrophomina root rot (Fig. 4). The greatest stem diameter was in treatment with B17B and B17A in Expt. 2 and B17A in Expt. 3, thereby reinforcing the impact of these bacterial BCAs on disease control and subsequently on plant growth. Similarly, the oven dry weight of plants treated with B17A, B17B, and control treatment with no *M. phaseolina* or BCA was significantly higher than all other BCAs and the fungicide treatment (Fig. 5). These results suggested that macrophomina root rot impacted plant growth as well as powdery mildew severity in repeated experiments (Fig. 1B). The improved plant growth from bacterial BCAs may be attributed to a combination of their ability to control both root rot and powdery mildew severity. However, fungicide applications also controlled both powdery mildew and macrophomina root rot, but fungicide treated plants were smaller and less green than those treated with the bacterial BCAs. Hence, it is likely that the bacterial BCAs had another effect on the plants that may include improved nutrient uptake, or the production of growth hormones and/or other secondary metabolites that impacted plant growth.

Previous studies on bacterial BCAs (B17A and B17B) have demonstrated endophytic colonization of treated dogwood plants and their effects in suppressing powdery mildew severities (Mmbaga et al., 2016). Other studies on the interactions between endophytic bacteria and their host plants were directly linked with beneficial effects such as plant growth promotion and biocontrol activity against plant pathogens (Bashan et al., 1990; Hallmann et al., 1997; Pleban et al., 1995). Waller et al. (2005) reported that root colonization of barley by the endophytic fungus *Piriformospora indica* reduced the incidence of powdery mildew by inducing systemic resistance in the host plant. The beneficial effect on plant defense was detected in distal leaves, demonstrating a systemic induction of resistance by a root-endophytic fungus (Waller et al., 2005). In addition, Hardoim et al. (2008) reported that some endophytic bacteria promoted host plant growth by producing plant growth-promoting substances and fixing nitrogen (N) from the atmosphere (Sturz et al., 2000).

Although there were slight variations in best disease suppression in different experiments, overall, this study has shown that the presence of *M. phaseolina* in the soil can impact both powdery mildew severity and plant growth (Figs. 1, 4, and 5). BCAs B17A and B17B appear to act as biostimulants and, as such, have the potential to contribute to the minimization of root rot and powdery mildew. The two bacterial BCAs were initially isolated from flowering dogwood in the wild (Mmbaga et al., 2016), suggesting that in natural environments, beneficial microbial disease antagonists may benefit plant growth in different ways. While the results from this study have confirmed previous studies (Mmbaga et al., 2016) on the efficacy of bacterial BCAs B17A and B17B in suppressing powdery mildew in the greenhouse, this study has also demonstrated antagonisms against *M. phaseolina* in compounded infections. The significant effect of B17A and B17B in reducing the severity of both diseases and improving plant growth better than a conventional fungicide commonly used in dogwood nursery production suggests a need for more studies on their role as growth promoters as well as their mechanisms of action and efficacy on other root rot pathogens. Reports on an endophytic isolate of *Pseudomonas* sp. showed its capability in concurrent production of indole acetic acid and solubilization of inorganic phosphate (Bano and Musarrat, 2003; Oteino et al., 2015). The isolate was also associated with significant production of hydrogen cyanide and siderophores that are well documented for their role in biocontrol of soil borne pathogens (Leong, 1986). There is a need to better understand the role of the two BCAs in production of growth-promoting substances and/or their role in nutrient uptake or induced systemic resistance, as well as for analysis of secondary metabolites associated with these BCAs. In addition, field studies are also needed.

Conclusion

In this study, bacterial isolates B17A and B17B, yeast isolates Y14 and Y4, and fungal isolate F16 suppressed powdery mildew severity, an important disease problem in nursery production of dogwood. B17A and B17B demonstrated superior biocontrol activity by also reducing root rot from *M. phaseolina*. However, isolates Y14, Y4, and F16 were ineffective against macrophomina root rot and isolate F13 was ineffective on both powdery mildew and macrophomina root rot.

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