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Potential Biological Control Agents for Soilborne Fungal Pathogens in Tennessee Snap Bean Farms

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***Abstract*. Fungi isolated from snap bean roots and rhizosphere soil where fungicides are not used included *Fusarium oxysporum*, *Fusarium equiseti*, *Fusarium subglutinans*, *Fusarium camptoceras*, *Fusarium chlamydosporum*, *Fusarium verticillioides*, *Fusarium proliferatum*, *Fusarium acuminatum*, *Fusarium solani*, *Peyronellaea pinodella*, *Macrophomina phaseolina*, and *Glomerella guttata*. Only *P. pinodella*, *M. phaseolina*, and *F. oxysporum* were isolated on symptomatic plants. These soilborne fungi are common pathogens of diverse host plants. Pathogenicity tests under controlled environment demonstrated that these fungi were pathogenic on snap beans. Subsequently, bacterial endophytes isolated from snap bean roots, papaya roots and stems, and dogwood stems were evaluated as potential biological control agents against these diverse fungi. All bacteria isolated, including *Bacillus vallismortis* (PS), *Bacillus amyloliquefaciens* (Psl), *Bacillus subtilis* (Prt), *Bacillus thuringiensis* (Y and IMC8), *Enterobacter* sp. (E), *Stenotrophomonas* sp. (B17A), and *Serratia* sp. (B17B) suppressed growth of the fungal pathogens in vitro and formed clear inhibition zones in petri dish dual cultures. Growth media taken from the inhibition zones suppressed growth of the fungal pathogens in the absence of the bacterial cells, suggesting that the bacteria released unidentified antagonistic biochemical substances into the media. This study constitutes an initial screening of endophytes as biological control agents against diverse fungal pathogens and forms a basis for the discovery of novel strains that can be further developed and integrated into disease management systems for diverse fungal pathogens. Isolates *B. vallismortis* (PS), *B. amyloliquefaciens* (Psl), *B. subtilis* (Prt), and *B. thuringiensis* (Y IMC8) exhibited the best performance as potential biological control agents paving the way for larger-scale in vivo studies and characterization of their interactions with fungal pathogens.**

Fungal pathogens impose major constraints on agricultural production globally (Collinge et al., 2010). Disease management strategies have relied heavily on conventional chemical fungicides. Persistent challenges associated with the use of conventional fungicides include toxicity to humans and nontarget organisms, environmental pollution, and development of fungicide resistance (Barnard, 2010; Burns et al., 2013; Kelley et al., 2013; Norgaard and Cedergreen, 2010). Attempts to introduce eco-friendly microbial pesticides as biological agents for combating fungal pathogens have been hampered by the lack of consistent field results. This problem has been attributed to environmental fluctuations of temperature, moisture, and nutrient availability (Schisler et al., 2002), as well as harmful effects of ultraviolet light (Tamez-Guerra et al., 2000; Vorholt, 2012) and variable and sporadic pathogen explosions (Fernando et al., 2000; Francl et al., 1999). In addressing challenges imposed by fungal pathogens, there is a need to explore the use of novel, robust, and naturally abundant endophytes that colonize plants internally without causing harm to their host plants. Such organisms are likely to be less vulnerable to external environmental fluctuations and are more likely to be effective in the field environments. Endophytes are thought to protect their host plants from pathogens by producing bioactive metabolites that enhance plant defense systems against pathogens (Clay and Schardl, 2002; Webber, 1981).

Snap bean (*Phaseolus vulgaris*), the second most important vegetable in Tennessee after tomatoes, was selected for this study. The production of snap beans is also widespread across the United States, with Tennessee ranking fifth in production after Florida, Georgia, California, and New York (USDA/NASS, 2015). Snap beans are susceptible to various major soilborne pathogens such as *Pythium* damping-off, wilt, and pod rot (various *Pythium* species), *Rhizoctonia*, *Fusarium* (*F. solani* f. sp. *phaseoli* and *F. oxysporum f.* sp. *phaseoli*), *Phytophthora* spp., *Sclerotium rolfsii*, and *Macrophomina phaseolina* (Bost et al., 2013). Seed treatments with chemical fungicides have been useful in managing soilborne pathogens because these treatments protect plants during the seedling stage when they are most vulnerable and can eliminate the need for foliar fungicide applications later in the season. Although the practice is almost always effective, the chemical fungicides kill nontarget organisms that may provide natural protection to plants against pathogens. In addition, their nontarget environmental impacts include toxicity hazards to humans and animals consuming treated plants. The development of pathogen resistance to chemicals is an additional problem that has led to the search for alternative methods (Pertot et al., 2015). Furthermore, most soilborne fungal pathogens form resting structures, such as sclerotia, oospores, and chlamydospores, which have the ability to survive in the soil for many years and are very difficult to control (Bost, 2006; Bost et al., 2013). Biological control agents (BCAs) can be helpful in decreasing the soil inoculum potential of soilborne pathogens and therefore improve soil health and overall health of plants (Pertot et al., 2015).

Root rot diseases accounted for estimated losses of 30% in snap beans in Tennessee in 2002 (Bost et al., 2013). Most root rot damage occurs when plants are young and damage may remain minimal when plants are growing vigorously and conditions for disease problems are unfavorable. However, when environmental conditions favoring the pathogen persist through flowering, yield losses can approach 100%. Cultural practices have been useful in combating root rot diseases, and constitute the main method used in organic production systems. However, the practice does not provide adequate protection, resulting in high yield losses and high prices of organic produce. With increasing consumer awareness of toxicity hazards posed by fungicides, there is a growing demand for organic produce, and new products suited to organic production systems are needed not only to reduce losses and boost production, but also to reduce sale prices, especially in fruits and vegetables. The use of beneficial microorganisms as part of integrated disease management systems can complement cultural practices and improve disease management (Mmbaga et al., 2018a; Pal and Gardener, 2011).

Our previous studies identified beneficial bacteria that significantly suppressed powdery mildew disease severity in flowering dogwood (Mmbaga and Sauvé, 2009; Mmbaga et al., 2008, 2016). Some BCAs applied on the roots suppressed powdery mildew on dogwood foliage (Mmbaga et al., 2016), and were effective against *Macrophomina* root rot disease (Mmbaga et al., 2018b). The objectives of this study were 1) to identify soilborne pathogens from snap bean roots and rhizosphere soil where fungicides are not used in organically produced snap beans, and 2) to screen endophytic bacteria for bioactivity in

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suppressing growth of diverse soilborne fungal pathogens and identify novel strains that can be further developed as BCAs.

Materials and Methods

Isolation and identification of root rot pathogens and endophytic BCAs. Isolation of root rot pathogens in snap beans (*Phaseolus vulgaris*) was conducted at four different locations, including Tennessee State University (TSU) bean research fields, TSU outreach community gardens growing diverse horticultural crops including snap beans and other vegetables, and selected organic vegetable farms in Palmyra and Indian Mount, TN. Each farm was inspected for symptomatic plants that displayed growth stunting, wilting, or leaf yellowing/browning or necrotic lesions on the collar regions. Ten sample plants with different symptoms were collected and taken back to the laboratory for pathogen isolation. Soil from around the roots (rhizosphere soil) was also collected for the isolation of soilborne pathogens. Direct isolation of potential pathogens from the symptomatic plants was done by using small pieces of tissue in the root and collar regions. The small plant tissues were cleaned and surface disinfected by dipping in 10% NaOCl for 2 min and then rinsed twice in sterile water, blotted dry using heat-sterilized paper towels, and plated on acidified potato dextrose agar media (APDA) containing 0.1% lactic acid. Bacteria growing as endophytes inside healthy and asymptomatic plants were isolated from roots of beans, stems and leaves of papaya (*Carica papaya*), and stem of dogwood, *Cornus florida*. Small pieces of the plant tissues were disinfected by dipping in 10% NaOCl and plated on nutrient agar media (NA), as described previously.

Isolation of soilborne pathogens from rhizosphere soil was done by using a baiting technique in which rhizosphere soil was placed in large petri dishes and moistened with sterilized water, two plates for each sample. Based on our previous results in which surface-disinfected carrots, apple, pine needles, and leaves of *Pieris japonica* were most effective in the isolation of diverse soilborne pathogens from rhizosphere soil, these baits were selected and placed in the soil for 48 h and then aseptically removed, blotted dry using heat-sterilized tissue paper, and plated on APDA. Pure cultures were obtained by subculturing.

Pathogenicity tests on snap beans. A total of 27 fungal isolates that exhibited different morphological features were identified (Table 1). Of these, 16 isolates were from plant tissue and 11 isolates were from rhizosphere soil. The isolates were tested for pathogenicity on snap bean seedlings grown in clear plastic containers lined with moist paper towels and maintained in a growth chamber at 24 to 26 °C and 12-h fluorescent light. Plant inoculation with test isolates was done 7 d after sowing using 5-mm mycelial disks placed on the plant roots, one disk per plant with the mycelia touching the plant tissues; controls used clean media disks. Each isolate was tested on four plants arranged in a randomized complete block design. Development of root rot lesions was monitored and evaluated 14 d after inoculation when some roots were completely girdled with necrotic lesions. Re-isolation from the lesions was done and cultures were compared with the original inoculum to confirm Koch's postulates. The pathogenicity tests were repeated once.

Identification and characterization of fungal pathogens and endophytic biocontrol agents. Fungal isolates, confirmed as pathogens in pathogenicity tests, were microscopically observed and grouped into morphological types. Bacterial endophytes were observed and characterized using colony morphology, Gram staining, cell morphology, and DNA sequence analysis. The DNA of fungal pathogens and bacterial endophytes was extracted using FastDNA kit (MP Biomedical, Solon, OH) following the manufacturer's instruction manual. The concentration of the DNA and relative purity was checked using a Nanodrop Lite (Thermo Fisher Scientific, Wilmington, DE). Two universal polymerase chain reaction (PCR) primers, ITS1 (5′-TCC GTA GGT GAA CCT TGC GG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to amplify the ribosomal DNA (rDNA) from fungi. Primer pairs RW01 (5′-AAC TGG AGG AAG GTG GGG AT-3′) and DG74 (5′-AGG TGA TCC AAC CGC A-3′) were used to amplify a 370 base pair (bp) region of the 16S ribosomal RNA (rRNA) gene for bacterial isolates.

The PCR analysis of the genomic DNA was carried out in a final volume of 25 µL with PCR buffer 1X (Promega, Madison, WI), 2.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 100 pM of primer, 100 ng genomic DNA, and 2.5 units of Taq polymerase (Promega). Amplification was carried out using a PTC 100 Thermal Cycler (Boier Lifepro, Grand Island, NY) programmed with an initial denaturation temperature of 95 °C for 5 min, followed by 34 cycles consisting of denaturation at 95 °C for 1 min, annealing of the primers at 55 °C for 1 min, and 1-min initial extension at 72 °C followed by 10 min of final extension at 72 °C.

Twenty microliters of the PCR amplified products were separated on a 2% agarose gel (w/v) (Phenix, Candler, NC) alongside 100-bp ladder size standard to determine the size and quality of DNA fragments. GelRed stain (0.1 µg/mL) was used to aid nucleic acid visualization. Electrophoresis was performed at 50 to 60 V for 40 min in 1X Tris borate-EDTA buffer. The gels were photographed using Kodak Gel logic 200 (Carestream, Rochester, NY) under ultraviolet light. The PCR products were purified using the Exosap DNA purification kit (Invitrogen, Life Technologies, Inc., Carlsbad, CA) following the manufacturer's recommended protocols. The PCR products were sent to Eurofins genomics (Louisville, KY) for sequencing. Sequences were aligned using the online alignment tool BLAST. Identification of each endophyte was determined by comparing its DNA sequence with sequences previously deposited in GenBank (NCBI) using the closest similarity match at ≥99% identity.

Isolates that matched *B. thuringiensis, Bacillus cereus*, and *Bacillus anthracis* were examined for the presence of parasporal crystals that are associated with *B. thuringiensis* (Ejiofor and Johnson, 2002). Isolates were incubated for 72 h on medium containing yeast extract, $(NH_4)_2\ SO_4$, $K_2HPO_4.3\ H_2O$, $MgSO_4.7\ H_2O$, $CaCl_2.2\ H_2O$, $MnSO_4.4\ H_2O$, and glucose. A differential crystal staining technique was then conducted following the protocol described by Ejiofor and Johnson (2002). The presence of crystal proteins produced during sporulation is solely characteristic of *B. thuringiensis* and not the related bacteria, *B. cereus or B. anthracis*, which are human pathogens.

Screening of endophytes as BCAs of fungal pathogens. Eight bacterial endophytes including five presented in Table 2 and three (B17A, B17B, and IMC8) previously isolated from *C. florida* stem pieces and found to suppress *Erysiphe pulchra* (Mmbaga et al., 2018b; Rotich et al., 2019), were evaluated for antimicrobial activity against 11 soilborne fungal pathogens presented in Table 1 and *Glomerella acutata* previously isolated as a soilborne pathogen. The antimicrobial activity was assessed using a dual culture technique in which bacterial endophytes and fungal pathogens were placed at opposite sides of the petri plates containing both potato dextrose agar (PDA) and NA at 1:1 v/v ratio to support growth of both the fungal pathogens and the bacterial endophytes. Initial inoculum consisted of mycelial plugs of fungal pathogen (5-mm radius) from 7-d-old cultures grown on PDA, and 5-mm plugs of bacterial endophytes collected from 24-h cultures grown on NA. The controls consisted of fungal pathogens alone and endophytes alone. All plates were incubated at ambient temperature of 20 to 23 °C. The effects of bacterial endophytes on growth of the fungal pathogens were measured after 7-d growth. Percentage growth inhibition (PGI) was calculated using the formula: PGI (%) = (GC – GE)/GC × 100 in which GC = growth of fungal pathogen control plate and GE = growth of fungal pathogen in the presence of the endophytic bacteria. Each isolate was evaluated with four replicates, and the study was repeated once.

The data obtained were subjected to analysis of variance using SAS 9.4 software. Mean values among treatments were compared by using the least significant difference (LSD) test at $P = 0.05$. Mean comparisons were conducted using Fisher's LSD at $P \leq 0.05$.

Biochemical substances from endophytes as potential mechanisms of antagonism to fungal pathogens. This study was conducted to assess the presence of antagonistic diffusible metabolites in the media at the inhibition zone between the endophyte and the fungal pathogens. To ensure that the media from the inhibition zone did not contain any bacterial cells, 5.0-mm plugs of the media were plated on NA and monitored for growth of bacteria. Subsequently, bacteria-free 5.0-mm plugs

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Table 1. Soilborne fungi isolated from snap bean roots and from their rhizosphere soil in Tennessee.

Sample ID	Source	Organism identity	Accession no.	Percent identity	Necrosis incidence[z]
PA-SN1-L2	Snap bean	*Peyronellaea pinodella*	KM030324.1	100	20
PA-SN3-L1	Snap bean	*Fusarium oxysporum*	KF494076.1	100	30
PA-SN1-R1	Snap bean	*F. oxysporum*	KP942940.1	100	28
PA-SN4-R1	Snap bean	*F. oxysporum*	KM486071.1	99	28
JJ5	Soil	*F. oxysporum*	FJ605247.1	99	35
JJ5	Soil	*Fusarium equiseti*	AB425996.1	99	20
JJ8	Soil	*Fusarium subglutinans*	JN646040.1	100	30
JJ8	Soil	*Fusarium camptoceras*	EU520082.1	99	20
JJ9	Soil	*Fusarium chlamysosporum*	KM076600.1	99	20
JJ9	Soil	*Fusarium verticillioides*	KC752592.1	100	25
JJ10	Soil	*Fusarium proliferatum*	KJ608094.1	99	17
JJ11	Soil	*Fusarium acuminatum*	KF887088	99	17
JJ15	Soil	*Fusarium solani*	HQ262512	99	20
J116	Soil	*Macrophomina phaseolina*	JX945170	100	80

[z]Incidence of root necrosis in pathogenicity tests on snap beans varied from 17% to 80% of roots showing root rot lesions with symptoms of brown to reddish-brown lesions.

Table 2. Molecular identification of endophytes isolated from different hosts and their identity based on closest similarity match in DNA sequence with GenBank accessions.

Sample ID	Source	Organism identity	Percentage identity	Accession no.
PS	Papaya stem	*Bacillus vallismortis*	99	KJ642605.1
Prt	Papaya root	*Bacillus subtilis*	99	AB894357.1
Psl	Papaya stem	*Bacillus amyloliquefaciens*	99	KU551259
E	Snap bean root	*Enterobacter* sp.	100	KJ526911.1
Y	Dogwood stem	*Bacillus anthracis*	99	CP010852.1
		Bacillus thuringiensis[z]	99	CP010577.1
		Bacillus cereus	99	CP010577.1

[z]Confirmed identity based on Ejiofor and Johnson (2002).

from the inhibition zone were evaluated for the ability to suppress growth of the fungal pathogens by using dual cultures in which a pathogen plug was placed at the center of the 85-mm petri dish and four 5-mm plugs of bacteria-free agar from the inhibition zones were placed at equidistant positions (2.75 cm) from the pathogen plug. The control consisted of one 5-mm pathogen plug at the center of the 85-mm petri dish and media plugs from noninoculated NA at equidistant positions from the pathogen. Treatments were replicated four times. All plates were incubated at 25 ± 2 °C, arranged in a randomized complete block design for 14 d. Growth of fungal pathogens was measured and the pathogen growth inhibition by bacteria-free plugs was calculated as explained previously. The experiment was repeated once.

A second study was conducted to ascertain if potent biochemical substances released by the BCAs can pass through a 0.22-µm filter membrane (Sigma Aldrich, St. Louis, MO) into the media and inhibit pathogen growth in the absence of the bacterial cells. Sterile membrane filters were overlaid on PDA/NA at the center of the 85-mm petri plate and the selected endophytes were placed at the center and allowed to grow for 24 h; the membrane bearing the bacterial culture was then removed from the petri plate and a 5-mm mycelial plug of a fungal pathogen (*M. phaseolina*) was placed at the center of the plate where the membrane had been laid. A control treatment consisted of clean NA media plug and the pathogen (*M. phaseolina*) placed at the center of the plate where the membrane had been laid on the plate. Plates were replicated four times and the percentage inhibition of colony growth of the fungal pathogen was measured and compared with the control treatment as described previously.

Results

Isolation and identification of root rot pathogens and endophytic BCAs. Only two pathogens, *P. pinodella* and *F. oxysporum*, were isolated from symptomatic plant tissue that displayed shoot wilting, seedling stunting, and brown root lesions (Table 1). Pathogenicity tests of *P. pinodella* and *F. oxysporum* on snap bean seedlings revealed root rot lesions that developed on all inoculated plants, but not in the noninoculated controls. The necrotic root lesions were brown to reddish brown in color and completely girdled the roots in 14 d after inoculation. The pathogenicity test of other fungi isolated from rhizosphere soil produced root lesions that were similar to those produced by *P. pinodella* and *F. oxysporum* with no distinct symptom separation of the different fungi. The re-isolation of fungi that were morphologically similar to the original inoculum confirmed Koch's postulate and indicated that the fungi were associated with the root lesions. The initial field symptoms of shoot wilting, seedling stunting, and brown root lesions can be considered nondistinct. Thus, it is possible that disease symptoms observed in the field may be a result of a disease complex from the two fungi or a disease complex that involved other fungi in the rhizosphere soil. Several fungi isolated from the rhizosphere soil, including *F. equiseti*, *F. subglutinans*, *F. camptoceras*, *F. chlamydosporum, F. verticillioides, F. proliferatum*, and *M. phaseolina*, have been reported to be pathogens in other plants (Table 1).

Of the different baits used to isolate fungi from the rhizosphere soil, leaf discs of *Pieris japonica* resulted in the isolation of the most numerous fungal isolates, followed by pine, carrot, and apples, respectively. Based on previous results, these baits were most effective in the isolation of diverse soilborne pathogens from rhizosphere soil. Morphological characteristics of various *Fusarium* species isolated from the rhizosphere soil were consistent with the identified species according to Nelson et al. (1983). Results from pathogenicity tests in growth chamber experiments showed that all the previously mentioned fungi were pathogenic on snap beans and produced similar symptoms consisting of brown to reddish-brown necrotic lesions on snap bean roots of test plants, whereas the PDA agar control did not cause symptoms. This observation further suggested that these fungi may have contributed to the seedling stunting, leaf blight, shoot wilting, and brown root lesions observed in the field.

Of the bacteria isolated as endophytes, different morphological types were recognized and identified. *Bacillus* strains were most frequently isolated, *Enterobacter* sp. being the only other genus (Table 2). Isolate PS from papaya stem had the highest similarity match to *B. vallismortis* at 99% (GenBank number KJ642605.1), isolate Prt from papaya roots with 99% similarity match with *B. subtilis* (GenBank number AB894357.1), and Psl from papaya leaves having a similarity match at 99% identity to two bacteria, *B. subtilis* (GenBank number HQ266666.1) and *B. amyloliquefaciens* (GenBank number KU551259.1). Isolate Y, endophytic in dogwood, matched three *Bacillus* species, *B. anthracis*, *B. thuringiensis*, and *B. cereus*, at 99% identity (Table 2). Staining in isolate Y revealed the presence of crystal proteins with spherical or ovoid morphology and confirmed that this isolate was *B. thuringiensis* and not *B. cereus* or *B. anthracis,* which do not form crystal proteins. Isolate E was identified as *Enterobacter* sp. at 100% identity (GenBank number KJ526911.1).

Screening of the endophytes as BCAs for diverse fungal pathogens. Results in dual culture experiments showed that the selected endophytes caused significant colony growth inhibitions to seven species of *Fusarium* and three other soilborne pathogens (Figs. 1 and 2). Different endophytes caused significant growth inhibitions against diverse fungal pathogens and differences in pathogen/endophyte interactions were significant at $P = 0.001$. Inhibition zones for *M. phaseolina* was highest, and the degree of growth inhibition by different endophytes is presented in Fig. 2. The effect of the selected endophytes in suppressing pathogen growth varied between pathogens (Figs. 1 and 2). All endophytes were effective in inhibiting growth of *Fusarium* species, especially *F. verticillioides* and *F. oxysporum* (Fig. 1). Isolates Y and IMC8, both *B. thuringiensis,* were the most effective in inhibiting *Fusarium* species,

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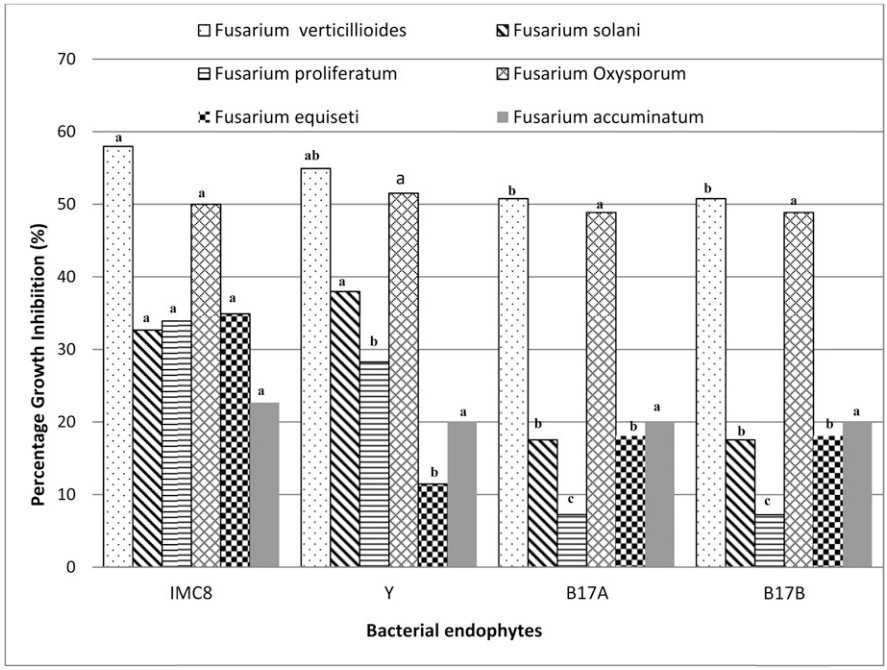

Fig. 1. The performance of bacterial endophytes in suppressing mycelial growth of diverse *Fusarium* species in dual cultures exhibiting their potential as biological control agents. Bacterial endophytes IMC8 (*Bacillus thuringiensis*); Y (*B. thuringiensis*); B17A (*Stenotrophomonas* sp.); and B17B (*Serratia* sp.). Different letters in each fungal pathogen (column patterns) indicate statistical differences at $P = 0.05$ according to SAS Prolog analysis.

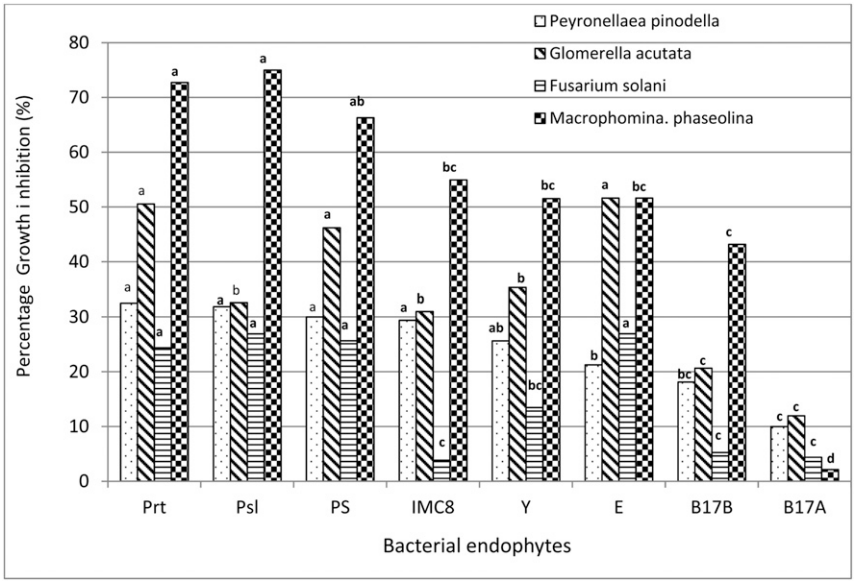

Fig. 2. The performance of bacterial endophytes in suppressing mycelial growth of diverse fungal pathogens in dual cultures indicating their potential as biological control agents. Bacterial endophytes PS (*Bacillus vallismortis*); Prt (*Bacillus subtilis*); Psl (*Bacillus amyloliquefaciens*); Y (*Bacillus thuringiensis*) and E (*Enterobacter*), B17A (*Stenotrophomonas* sp.), B17B (*Serratia* sp.), IMC8 (*B. thuringiensis*). Different letters in each fungal pathogen (column patterns) indicate statistical differences at $P = 0.05$ according to SAS Prolog analysis.

whereas isolates Prt, Ps, and Psl were most effective against *M. phaseolina* and B17A (*Stenotrophomonas* sp.), and B17B (*Serratia* sp.), being least effective (Fig. 2).

Assessment of diffusible metabolites as potential mediators of BCA antagonism. Using agar plugs from the growth inhibition zone, free of bacterial cells, caused growth inhibition of pathogens (Fig. 3A–C). The use of growth media that was used to grow the BCA on bacterial filter membranes caused even more growth inhibition of *M. phaseolina* (Fig. 3D and E; Table 3). These observations again suggest the presence of pathogen-inhibiting diffusible metabolites produced by the endophytes into growth media. Although the presence of bacterial cells was most effective in suppressing fungal growth, growth inhibition was also observed in the absence of bacterial cells.

Discussion

Most fungi isolated from the snap bean fields not treated with chemical fungicides were *Fusarium* species. Most of the fungi were isolated from the rhizosphere soil and not from plant roots. Similar observations were reported by Avanzato and Rothrock (2010). It is possible that the *Fusarium* species may be weak pathogens of snap beans in natural environments. The fungi isolated from the rhizosphere soil (but not from snap bean roots) produced necrotic lesions on snap beans in growth chamber conditions. It is possible that the growth chamber environment used for pathogenicity tests was more favorable for disease development than the field environment where the fungi were isolated. However, the presence of these pathogenic fungi in snap bean rhizosphere soil and the proven pathogenicity of these fungi on inoculated beans have implications regarding their potential threat when conditions favor plant infection or when another favorable host is grown. Most of the *Fusarium* species isolated in snap bean rhizosphere soil are well known and widespread pathogens of corn, causing stalk and ear rot worldwide (Nelson et al., 1981, 1983). The presence of these fungi in snap bean rhizosphere soil in organic production fields may be significant not only as potential root rot pathogens, but also as toxin-producing fungi and potential contaminants of produce from these fields. Example, *F. subglutinans* has been reported to produce disease symptoms such as shoot and leaf blight, shoot wilting, seedling stunting, heavy colonization of coleoptiles, and node damage on corn plants, but had no effect on seedling emergence and survival (Aboul-Nasr and Obied-Allah, 2013). Although *F. subglutinans* was only isolated from snap bean rhizosphere soil and not from plant tissues, the symptoms observed in field plants in our study could have been partly caused by *F. subglutinans*. Other reports show that *F. subglutinans* is a distinct species in the *Gibberella fujikuroi* species complex and a pathogen of *Pinus patula* seedlings. Similarly, *F. verticillioides* (syn. *F. moniliforme*) were isolated from soil, but not from plant tissue. Other species isolated from bean rhizosphere soil, such as *F. camptoceras, F. acumintum,* and *F. equiseti, F. chlamydosporum F. solani, F. proliferatum, Fusarium graminearum*, and *F. oxysporum,* have widespread distribution, survive a long time in crop debris and soil, and may have been pathogens in a previous crop. However, *F. oxysporum* was also isolated from symptomatic snap beans. Because most of these *Fusarium* species are toxin-producing and have the ability to produce and accumulate mycotoxins in infected tissues, they potentially endanger humans and animals, subsequently causing food rejections (Aboul-Nasr and Obied-Allah, 2013; Asran and Buchenauer, 2002; Nelson et al., 1993). The ability of the selected endophytic bacteria to suppress growth of toxin-producing *Fusarium*

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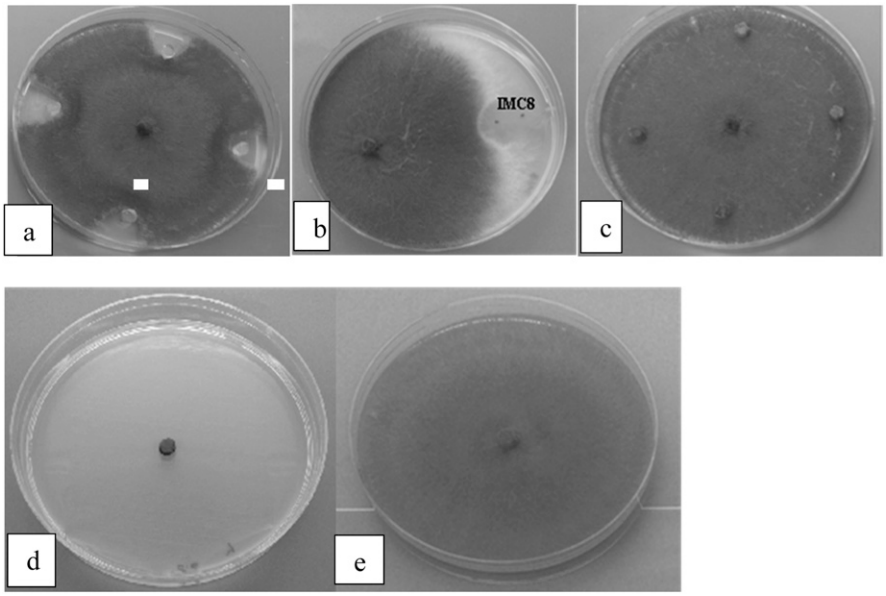

Fig. 3. Effect of diffusible metabolites released by different *Bacillus* species into growth media inhibiting *M. phaseolina* mycelial growth in culture in the absence of bacterial cells: (**A**) metabolites from the Prt (*Bacillus subtilis*) inhibition zone, (**B**) metabolites from the IMC8 (*Bacillus thuringiensis*) inhibition zone, (**C**) control treatment from plain nutrient agar (NA), (**D**) 100% growth inhibition of *Macrophomina phaseolina* from biochemical substances from isolate Y (*B. thuringiensis*) grown on a 0.22-µm filter membrane overlaid on potato dextrose agar (PDA)/NA media for 24 h and then removed compared with (**E**) mycelial growth of *M. phaseolina* on plain PDA/NA.

Table 3. Growth inhibition of *Macrophomina phaseolina* colonies in dual culture by bacteria-free media plugs taken from inhibition zones: 1) horizontal diffusion of metabolites released by endophytes and 2) vertical diffusion through filter membranes overlaid on the media.

		Growth inhibition of *M. phaseolina* colonies in the presence of diffusible metabolites		
Bacterial endophytes[z]	% growth inhibition[y]	Bacteria-free plugs from inhibition zone[x,w]	Bacteria-free plugs underneath filter membrane[x,w]	Bacterial cell plugs[y]
IMC8	54.93 bc	+	++	++
Y	51.52 cd	+	++	++
E	51.63 cd	+	++	++
Prt	72.73 a	++	++	+++
PS	66.29 ab	+	+	+++
Psl	75.00 a	+	+	+++
Control	0	–	–	NA
$LSD_{(0.05)}$	11.91			

[z]Endophytes IMC 8 and Y = *Bacillus thuringiensis*; PS (*Bacillus vallismortis*); Prt (*Bacillus subtilis*); Psl (*Bacillus amyloliquefaciens*); E (*Enterobacter*), and Control (plain media). LSD = least significant difference. No inhibition (–), slight inhibition of M. phaseolina (+), good inhibition (++), best inhibition (+++). NA = not applicable, no bacterial cells.
[y]Bacterial cells are present.
[x]Horizontal diffusion of metabolites from bacterial endophytes.
[w]No bacterial cells present. Numbers followed by different letters are significantly different at $P = 0.05$.

species has significant implications beyond the field production of snap beans, and may be a resource in the management of toxin-producing *Fusarium* species. Results from this study identified isolates of *Bacillus* species that have previously been documented to have an inhibitory effect on fungal pathogens (Hallmann et al., 1997; Heydari and Pessarakli, 2010). The isolation and identification of these new isolates presents potential new sources of BCAs for diverse fungal pathogens, including toxin-producing *Fusarium* species. Significant differences in growth inhibition by different endophytes against diverse fungal pathogens ($P = 0.001$) suggest that mixtures of endophytes could be used in plant protection for optimal control.

The DNA results of isolate Psl revealed its identity as either a *B. amyloliquefaciens* or *B. subtilis* strain, with both having 99% identity, and so it was difficult to distinguish them by molecular techniques. This difficulty was also reported by Hutsebaut et al. (2006), in which *B. subtilis* and *B. amyloliquefaciens* showed more than 99% similarity. They reported insufficient dissimilarity in the species of *B. subtilis* when pairwise alignment of 16S rRNA gene sequences analysis was done (Hutsebaut et al., 2006). Although the use of 16S rDNA sequence analysis is very common in assigning strains into different taxa, it is difficult to detect any variation when distinguishing closely related organisms (De Vos, 2002; Fox et al., 1992). The endophytes matched to members of the *B. subtilis* group pose particular identification problems: the *B. subtilis* group consists of *B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, *Bacillus mojavensis*, *B. vallismortis*, *Bacillus clausii*, *Bacillus atrophaeus*, *B. amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus sonorensis*, *Bacillus firmus*, *Bacillus lentus*, and *Bacillus sporothermodurans* (Standards Unit, Microbiology Services and PHE, 2015). Several studies have been conducted attempting to distinguish *Bacillus* species based on different phenotypic characteristics, such as fatty acid composition, pigmentation (Roberts et al., 1996), and analytical profile index (Logan and Berkeley, 1984). Thus, confirmatory identities of these endophytes require more studies using whole-genome DNA sequence analysis. The presence of crystal proteins (Ejiofor and Johnson, 2002) indicated that our isolates Y and a previously isolated endophyte of flowering dogwood IMC8 are strains of *B. thuringiensis*. The effect of *B. thuringiensis* isolates Y and IMC8 in suppressing growth of fungal pathogens is interesting in that *B. thuringiensis* is a well-known insect pathogen widely used in commercial biological control. Our recent studies showed that IMC8 has suppressed powdery mildew disease severity in *C. florida* (Rotich et al., 2019). *Bacillus* isolates Psl, PS, and Prt suppressed phytophthora root rot in bell pepper (Irabor and Mmbaga, 2017) and soybean root rot caused by *F. oxysporum* and *Fusarium graminearum* (Zhang et al., 2009); their ability to suppress diverse fungal pathogens increases their prominence as potential BCAs.

The bacterium *Enterobacter* sp. was isolated as an endophyte of snap bean roots from a grower's farm in Tennessee; however, *Enterobacter* spp. is present almost everywhere in nature, particularly in the rhizosphere and spermosphere of most plant species (Haahtela et al., 1981; Ladha et al., 1983). Some *Enterobacter* spp. have been reported to promote plant growth (Deepa et al., 2010; Ramesh et al., 2014), associated with nitrogen fixing in rice (Ladha et al., 1983) and control some plant diseases (Howell et al., 1988; Mmbaga and Joshua, unpublished). Isolates B17A (S*tenotrophomonas* sp.) and B17B (*Serratia* sp.) have previously demonstrated disease control against powdery mildew and growth-promoting abilities in *C. florida* (Mmbaga et al., 2018b) and soybeans (Wahyudi et al., 2011).

Numerous bacterial strains have been shown to protect plants against pathogens and promote growth in different ways, such as inhibition, competition, or increasing plant resistance (Idris et al., 2007; Richardson et al., 2009). Several species, such as *B. subtilis*, *B. licheniformis, Bacillus pumilus*, *B. amyloliquefaciens*, *B. cereus*, and *B. thuringiensis*, have been reported to suppress growth of various fungal pathogens, such as *Rhizoctonia*, *Fusarium*, *Sclerotinia*, *Sclerotium*, *Gaeumannomyces*, *Nectria*, *Pythium*, *Phytophthora,* and *Verticillium* species (Basurto-Cadena

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et al., 2012; Haleem Khan et al., 2011; Zhang et al., 2009).

The screening of bacterial endophytes in our in vitro studies showed clear inhibition zones. The use of dual cultures is a rapid and convenient method to evaluate endophytes as potential BCAs against plant pathogens before testing them on whole plants. Our studies are supported by the approach of Islam et al. (2005), who used the existence of inhibition zones as criteria in screening and selecting potential BCAs. Our studies showed that the inhibition zones harbored unidentified compounds, diffused into the media. Such compounds inhibited fungal growth in the absence of bacterial cells. Although the compounds have not yet been identified, results showed clearly that bioactive metabolites were effective in suppressing fungal pathogens. This agrees with reports by Clay and Schardl (2002), Webber (1981), Raaijmakers et al. (2002), and Heydari and Pessarakli (2010) that bacterial antagonists inhibit the growth of fungal pathogens by excreting antifungal metabolites, such as antibiotics, toxins, and bio-surfactants, including volatiles. The diffusion of the compounds to the media where bacteria were grown on filter membranes displayed greater inhibition, perhaps because of higher concentrations where the bacteria were grown on the micro filter membranes (Fig. 3).

Differences in growth inhibition between the isolates also suggested that different bacterial endophytes may produce different compounds and/or amounts of antifungal substances. However, these differences in the antagonistic effect against different pathogens suggest a possibility for using mixed populations of endophytes to maximize control of fungal pathogen complexes. Isolates B17A and B17B, previously isolated as epiphytes of flowering dogwood, suppressed powdery mildew disease severity and promoted plant growth in dogwood plants even when applied on the roots (Mmbaga et al., 2016). The isolates also colonized dogwood endophytically and were antagonistic to *Macrophomina* root rot in this host (Mmbaga et al., 2018b). Notwithstanding, this study showed that B17A and B17B were most effective against *F. oxysporum* and *F. verticillioides* and less effective against other soilborne fungi tested, including *M. phaseolina* (Fig. 3). These results suggest that there is some host specificity as well as pathogen/endophyte specificity in bioactivity of these endophytes. Endophytic organisms used in this study are likely to be naturally abundant and less vulnerable to external environmental fluctuations, and therefore more likely to confer effective disease control in field environments. Such BCAs can be integrated with other cultural strategies, including sanitation and crop rotation in a biological-based integrated disease management system for maximized disease control in organic farming.

Our results showed that the extent of growth inhibition resulting from plugs taken from bacteria-free inhibition zones was greatly reduced compared with plugs containing the bacterial cells (Table 3). The pathogen inhibition evident in the absence of the bacterial cells indicates that the selected endophytes may not have to be physically present for pathogen inhibition to occur, but the inhibition increased when bacterial cells were present. These observations suggest that more than one mechanism of action may be involved. Manipulating such organisms to be ''super producers'' of antagonistic metabolites may improve their efficacy. Observations from this study create considerable potential for the development of disease management products that may be compatible with organic production; however, more studies are needed to identify and characterize the secondary metabolites and test the organisms in vivo.

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