

Evaluation of Selected Bacterial Endophytes for Biocontrol Potential against Phytophthora Blight of Bell Pepper (*Capsicum annuum* L.)

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Abstract

Phytophthora blight, caused by *Phytophthora capsici*, is the most destructive disease of bell pepper in the United States. The effectiveness of current management strategies is limited by the long-term survival of oospores, wide host range, aggressive fungicide-resistant isolates, and lack of resistant cultivars with acceptable agronomic traits. Biological control is a viable alternative, and the use of endophytic microorganisms as biological control agents (BCA) has attracted widespread attention because they colonize the same ecological niches as plant pathogens. Seven isolates of bacterial endophytes in three genera *Serratia* (B17B), *Enterobacter* (E), and *Bacillus* (IMC8, Y, Ps, Psl and Prt), isolated from papaya, snap bean and flowering dogwood were evaluated for effects on *P. capsici* mycelial growth *in vitro*, and on disease severity and plant growth in greenhouse environment. All isolates significantly inhibited the mycelial growth of *P. capsici* with Ps, Psl and Prt having superior inhibitory effects. Seed treatments with Ps, Psl and Prt followed by plant inoculation with *P. capsici* reduced disease severity, and significantly increased plant shoot height, fresh weight, and fruit yield (number and weight) with Ps and Prt being slightly superior to Psl. Compatibility tests between isolates showed that only B17B and Y were compatible with each other. Sensitivity tests to different levels of mefenoxam (Ridomil Gold SL) showed that Ps, Psl and Prt were tolerant to the fungicide, while *P. capsici* was highly sensitive. Thus, Ps, Psl and Prt can be used in rotation with mefenoxam to reduce the frequency of fungicide usage for a more environmentally friendly, long lasting, consistent, and effective control of phytophthora blight. Overall, all isolates tested are potential BCAs against *P. capsici*, but more greenhouse and field studies are required to confirm *in vitro* results for all isolates that showed good potential in *in vitro* studies.

Keywords: *Phytophthora capsici*; Soil-borne pathogens; Fungicide resistance; Biological-based IPM; Vegetable diseases

Introduction

Bell pepper (*Capsicum annuum* L.) is one of the most important vegetable crops in the world [1-4]. The crop thrives best under warm climatic conditions which also favor the development of many plant diseases. Disease constitutes one of the major constraints affecting commercial pepper production in the United States. Phytophthora blight, caused by *Phytophthora capsici*, is a widespread and highly destructive disease of peppers in tropical, subtropical, and temperate regions of the world [5]. It is especially important in the southern and southeastern United States where warm temperatures, high relative humidity and frequent rainfall promote rapid disease development [3].

In recent years, Phytophthora blight has become the most prominent disease of peppers in the United States [3,6]; total crop losses from *P. capsici* root rot can occur under wet conditions [6]. The wide host range of *P. capsici* consists of more than 45 species of crops and weeds belonging to 14 families [7-10]. The pathogen is a heterothallic, soil-borne oomycete (water-mold) characterized by asexually produced motile, biflagellate zoospores and sexually produced thick-walled oospores. The pathogen can survive for several years in the soil in the form of oospores even in the absence of susceptible crops and initiate infection when a host plant becomes available. The pathogen infects the entire plant causing symptoms such as foliar blight, stem canker, fruit, crown, and root rots [3,7,11]. Disease development is favored by warm temperatures, high rainfall or irrigation, and poor soil drainage [8]. The disease is managed mainly by crop rotation, application of chemical fungicides such as metalaxyl or mefenoxam, and management of irrigation water [12-15]. However, it is difficult to manage Phytophthora diseases because of the long-term survival of oospores in the soil, wide host range, and long-distance movement of the pathogens through soil and water [6,7]. Lack of resistant crop varieties with acceptable agronomic traits [16,17], and the rise of new aggressive phytophthora

strains resistant to chemical fungicides [6] are additional challenges for the management of phytophthora blight in vegetables. Furthermore, the indiscriminate use of chemical pesticides is raising concerns due to their toxicity hazards on human health and the environment [18,19]. These concerns have led to the phase-out of some chemical fungicides such as the ozone-depleting soil fumigant methyl bromide [20]. Therefore, there is a need for more effective, sustainable, and environmentally friendly management strategies for phytophthora blight.

Biological control is a viable alternative for plant disease management especially as a component of an integrated disease management program [19,21,22]. Biological control agents antagonize pathogens directly by hyperparasitism, predation, and production of antibiotics and lytic enzymes; and indirectly by competing for space and nutrients, inducing systemic resistance, and promoting plant growth [18,19,23,24]. Several fungal and bacterial BCAs have been shown to be effective against many pathogens of peppers [16,25]. However, commercially available biocontrol agent for the management of Phytophthora blight are needed; continued search for effective BCAs will benefit crop production and especially benefit organic farming systems. The success of biological control is often dependent on the ability of the BCA to colonize the host

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plant tissues [18,26,27] and endophytes may provide valuable natural resources.

Endophytes are fungi and/or bacteria which asymptotically inhabit the internal tissues of plants [28]; they colonize the same ecological niches as disease-causing organisms [29,30]. Many are known to form mutually beneficial associations with their hosts and play roles essential in plant survival; some promote plant growth, induce plant defense mechanisms, and reduce disease severity, protect plants against herbivory, fix atmospheric nitrogen and increase mineral nutrient uptake [31-34]. Endophytes are ubiquitous, and every plant studied to date harbors at least one or more endophytic microbial species [30,33,35]. Antagonistic effects of bacterial endophytes on various pathogens of agriculturally important crops have been demonstrated [36-38]. However, the complexity of microbial interactions in natural environments is not well understood and search for biocontrol agents that are adequately effective in field applications remains important.

The objectives of this study were to screen endophytes isolated from flowering dogwood, snap bean, and papaya for biocontrol activities against *P. capsici*, evaluate the compatibility between bacterial endophytes, and compatibility of selected isolates with fungicide mefenoxam that is commonly used to control *P. capsici*.

Materials and Methods

Pathogens, bacterial isolates, and culture conditions

Isolates of *P. capsici* from infected pepper plants were previously isolated using PARP (pentachloronitrobenzene, ampicillin, rifampicin and pimarinic)-amended V8 juice agar media [39] and maintained on potato dextrose agar (PDA) or clarified V8[®] juice agar media (cV8, 800 ml distilled water, 200 ml of clarified V8 juice, 2 g of CaCO₃, and 15 g of bacteriological agar); short-term culture storage was done at 4 ± 2°C. *Phytophthora* cultures used in this study were 7 to 10 days-old, grown in PDA or V8 agar at 28 ± 2°C. Bacterial endophytes used in this study (Table 1) were previously isolated from flowering dogwood, snap bean and papaya, maintained on nutrient agar (NA) or Luria-Bertani (LB) agar media, and stored at -80°C; fresh 24 h-old cultures grown at 28 ± 2°C were used for this study.

In vitro evaluation of bacterial endophytes for bioactivity against *P. capsici*

Dual culture assays were conducted to evaluate the inhibitory effects of the endophytes on the mycelial growth of *P. capsici* on nutrient agar (NA) and Luria-Bertani (LB) agar. Growth media for dual culture assays consisted of equal parts of PDA and NA or LB to facilitate the growth of the pathogen as well as the bacterial endophyte. A 5-mm mycelial plug cut from the actively growing margins of *P. capsici* cultures grown on PDA was placed at the centre of an 85-mm Petri dish containing PDA-NA/LB (1:1) growth media. Four 5-mm plugs of the test endophyte grown on LB or NA were placed at equidistant positions (2.75 cm) from the pathogen. Pathogen control plates consisted of the pathogen plug

with LB or NA plugs, while the BCA control plates consisted of four BCA plugs with a PDA plug. Each treatment had four replicates. The plates were placed in an inverted position in an Isotemp[™] incubator (Fisher Scientific[™], Waltham, MA 02451, USA) at 25 ± 2°C and observed for mycelial growth. At day 7, the mycelial radius was measured and *P. capsici* growth inhibition was calculated using the formula;

$$\% \text{ Growth inhibition} = R_2 - R_1 / R_2 \times 100$$

where, R₂ = *P. capsici* mycelial radius in control plate (cm), R₁ = *P. capsici* mycelial radius in pathogen-BCA dual culture (cm)

Compatibility between bacterial endophytes

To determine isolate combinations that have potential for additive and synergistic effects, compatibility between any two endophytes was determined by conducting a modified agar diffusion (or Kirby-Bauer) test. A loopful of 24 h old culture grown in LB agar was transferred to glass test tubes containing 5 ml of LB broth and incubated for 24 h on an incubator shaker (New Brunswick Scientific CO., Inc, Edison, NJ 08817, USA) set at 200 rpm and 30°C. The concentration of the bacterial suspension was then quantified and adjusted to ~10⁸ CFU/ml. Sterile 8-mm Whatman number 1 filter paper discs were dipped aseptically into the media suspension of the test endophyte (endophyte 1), and air dried for 30 min. The other endophyte (endophyte 2) was swabbed uniformly on the entire plate using a sterile cotton-tipped applicator. Four discs impregnated with bacteria suspension of endophyte 1 were gently pressed onto the endophyte 2 plated agar surfaces at four equidistant positions using sterile forceps. The plates were incubated at 28 ± 2°C and observed over a period of 72 h and incompatible endophytes were identified by a zone of inhibition between them.

Evaluation of bacterial endophytes and *Phytophthora capsici* isolate for sensitivity to mefenoxam

Three isolates Ps, Psl and Prt that gave best performance on *in vitro* studies were assessed for sensitivity to mefenoxam by using the turbidometric method. Isolates were prepared in LB broth as described above; 100 µl of bacterial suspension was transferred to test tubes containing 5 ml of LB broth previously amended with mefenoxam (Ridomil Gold SL, 480 mg a.i ml⁻¹) at concentrations of 0, 10, 100, or 1000 µg ml⁻¹. Each fungicide concentration was replicated four times for each isolate. Test tubes were incubated at 30°C in an incubator shaker (New Brunswick Scientific CO., Inc, Edison, NJ 08817, USA) set at 200 rpm for 24 h under ambient light and their concentrations were measured as optical density at 600 nm.

The *P. capsici* isolate was also tested for sensitivity to mefenoxam using the method of Parra and Ristaino [40]. Mycelial plugs (5 mm in diameter) of the *Phytophthora* isolate was obtained from the actively growing margins of a 7-day culture maintained on V8 agar medium and transferred to V8 media amended with mefenoxam (Ridomil Gold SL, 480 mg a.i ml⁻¹) at concentrations of 0, 5, 10 and 100 µg ml⁻¹ with four replicates for each fungicide concentration. Petri dishes were incubated in an inverted position at 25°C for 7 days in ambient light. At day 7, the mycelial diameters were measured and compared to diameters from non-amended control plates.

Characterization of isolates as sensitive, intermediate, or resistant was based on isolate growth on media amended with different concentrations of mefenoxam as compared to non-amended media. The isolate was regarded as sensitive if the mycelial growth on media amended with 5 µg ml⁻¹ of mefenoxam was less than 40% of the growth on non-amended media; intermediate, if growth on media amended with 5 µg ml⁻¹ was greater than 40% of that on nonamended media, but

Isolates	Endophytes	Plant source
B17B	<i>Serratia marcescens</i>	Dogwood stem
E	<i>Enterobacter</i> sp	Snap bean root
IMC8	<i>Bacillus thuringiensis</i>	Dogwood stem
Y	<i>B. thuringiensis</i>	Dogwood stem
Ps	<i>B. vallismortis</i>	Papaya stem
Psl	<i>B. amyloliquefaciens</i>	Papaya stem
Prt	<i>B. subtilis</i>	Papaya root

Table 1: Bacterial endophytes and their sources.

growth on media amended with 100 µg ml⁻¹ was less than 40% of that on nonamended media; if growth on media amended with 100 µg ml⁻¹ was greater than 40% of that on non-amended media the isolate was characterized as resistant [40].

Pathogenicity test of *P. capsici*

The *P. capsici* isolate used in this study was tested for pathogenicity on seedlings of bell pepper (*C. annuum* ‘California Wonder’) in the greenhouse. Surface sterilized pepper seeds were sown in 10-cm² plastic pots containing heat-sterilized Miracle-Gro® potting mix. Plants were fertilized twice a month using Miracle-Gro® Water Soluble All-Purpose Plant Food starting two weeks after seedling emergence. Inoculation with *P. capsici* was done eight weeks after germination by drenching the base of each plant with 25 ml zoospore suspension (10⁷ zoospores/ml) and control plants were drenched with 25 ml of sterile distilled water. Plants were arranged in a randomized complete block design with six replicates per treatment and one plant per pot as a replicate. The study was repeated once.

Disease severity was evaluated beginning 2 days after inoculation using a scale of 0-5, in which 0 = no visible symptoms; 1 = Slightly wilted with brownish lesions beginning to appear on the stem; 2 = stem lesions extending to cotyledons and 30% of plant diseased; 3 = stem lesions extending to petioles and 50% of the plant diseased; 4 = petioles collapse and 80% of the plant diseased; 5 = entire plant dead. Re-isolation of the pathogen from diseased plants root, crown, stem, and leaf tissues was done using PARP-amended V8 media. Petri plates were incubated for 7 days at room temperature and colonies with growth characteristic of *P. capsici* were transferred to clarified V8 juice agar. The pathogen was identified based on colony morphology and sporangial characteristics; identification was confirmed using DNA sequence analysis.

Evaluation of bacterial endophytes for biocontrol of *P. capsici* in bell pepper seedlings

The study was conducted in a greenhouse at 25 ± 3°C. Bell pepper ‘California Wonder’ seeds were surface-sterilized and treated with the bacterial isolates presented in Table 1 using inoculum concentration of ~10⁸ colony forming units (CFU) per ml. Seeds were soaked in inoculum suspension for 1 h and non-treated control seeds were soaked for 1 h in sterile water. Treated seeds were sown in 10-cm² plastic pots containing heat-sterilized Miracle-Gro® potting mix and arranged in a randomized complete block design with six replicates of individual plants per replicate. Plants were fertilized with Miracle-Gro® Water Soluble All-Purpose fertilizer as described above. Eight weeks after germination, plants were inoculated with *P. capsici* and control plants were treated

Treatments (bacteria)	LB agar growth media		Nutrient agar growth media	
	Mycelial growth (cm)	% Growth Inhibition	Mycelial growth (cm)	% Growth Inhibition
B17B	1.27c*	60.89c	1.84g	51.59g
E	0.93b	71.41b	1.28d	66.27d
IMC8	1.19c	63.39c	1.43e	62.50e
Y	1.24c	61.94c	1.49f	60.69f
Ps	0.75ab	76.96ab	0.99b	73.92b
Psl	0.90ab	72.41ab	0.88a	76.78a
Prt	0.73a	77.55a	1.07c	71.86c
Control	3.25d	0.00d	3.81h	0.00h

*Means followed by the same letter are not significantly different at 5% level by Fisher's LSD

Table 2: Effect of bacterial endophytes on the mycelial growth of *Phytophthora capsici* grown on Luria-Bertani (LB) agar and on nutrient agar (NA).

with sterile distilled water as described above. Plants were monitored for disease development and severity using a 0-5 scale described above. The experiment was terminated 12 weeks after germination.

Effects of bacterial endophytes on plant growth and yield of bell peppers

To evaluate the effects of the bacterial endophytes on the growth and yield of seed-treated bell pepper plants, chlorophyll content, shoot and root length, plant fresh and dry weight, number of fruits per plant and total fruit weight were measured 12 weeks after germination. Chlorophyll content index (CCI) was measured using a handheld chlorophyll content meter (CCM-200 plus®, Opti-Sciences, Hudson, NH 03051, USA). Plant samples were dried to constant weight using Binder® Gravity Convection oven (Binder Inc., Bohemia, NY 11716, USA) at 70°C.

Statistical analysis

Statistical analyses were performed using IBM® SPSS Statistics® version 22. Data were subjected to analysis of variance (ANOVA) and mean comparisons were conducted using Fisher's least significant difference (LSD) at P ≤ 0.05. All experiments were repeated.

Results

Effects of bacterial endophytes on mycelial growth of *Phytophthora capsici* in LB and NA

All isolates tested significantly inhibited mycelial growth of *P. capsici* on both LB and NA (Figure 1). Isolates Ps, Psl and Prt caused the highest inhibition on *P. capsici* mycelial growth on both media with 76.96%, 72.41% and 77.55% growth inhibition on LB, and 73.92%, 76.78% and 71.86% growth inhibition on NA (Table 2). The growth media had some effect on the level of inhibition as well as on growth of the bacteria endophytes with E and IMC8 exhibiting higher inhibition on LB than on NA and larger colonies of E on NA than on LB resulting in a higher inhibitory effect on LB agar as compared to NA (Figure 1). Some isolates formed contact with the pathogen and some inhibition on LB media appeared to result from contact between the bacterial isolate and pathogen. While Ps and Prt showed higher inhibition on LB, Psl had higher inhibitory effect on NA (Figure 1).

Quantitative measurements of *P. capsici* mycelial growth and inhibition zones confirmed visual observations; all isolates significantly suppressed *P. capsici* mycelial growth, but the *Bacillus* isolates Ps, Psl and Prt displayed superior effect suppressing *P. capsici* growth by more than 70% on both LB and NA growth media (Table 2).

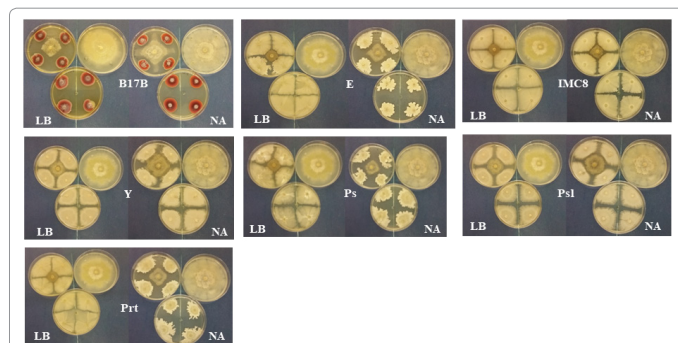


Figure 1: Dual culture assay between bacterial isolates and *Phytophthora capsici* on Luria-Bertani (LB) agar, and nutrient agar (NA) at day 7, showing significant inhibition of mycelial growth of the pathogen as compared to the control.

Pathogenicity test of *Phytophthora capsici* isolate on bell pepper

The *P. capsici* isolate used in this study was highly virulent on 'California Wonder' bell peppers. Dark lesions were noticed on the stem at the soil line within four days of inoculation. Stem lesions rapidly progressed up the plant followed by defoliation and plant death at approximately 10 days after initial disease symptoms were observed (Figure 2).

Effects of selected bacterial endophytes on *Phytophthora blight* severity in greenhouse.

Seeds that were not treated with the selected endophytes generated plants that were highly susceptible to *P. capsici* and showed the highest disease severity, with disease symptoms starting to develop within four days after inoculation with *P. capsici*; plants that were not inoculated with the pathogen did not develop any disease symptoms (Figure 3). Seed treatment with Ps, Psl and Prt reduced disease severities compared to the non-treated control. While all treatments suppressed disease severity, Prt was most effective in suppressing disease severity; Ps and Psl treated plants were not significantly different from Prt-treated plants, but they also were not significantly different from the non-treated control plants at $P \leq 0.05$ (Figure 3).

Effects of bacterial endophytes on plant growth and yield of bell pepper

Growth and development of plants as measured by different parameters showed significant differences in shoot height, plant fresh

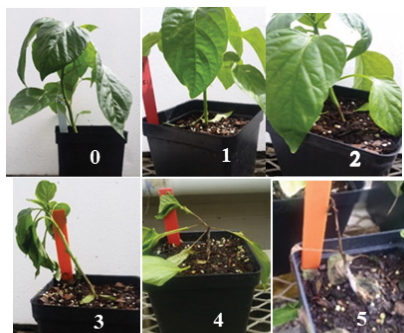


Figure 2: Different levels of *Phytophthora blight* disease symptoms. Disease severity of 0-5 scale in which 0 = no visible symptoms; 1 = Slightly wilted with brownish lesions beginning on the stem; 2 = Stem lesions extending to cotyledons and 30% of plant diseased; 3 = Stem lesions extending to petioles and 50% of the plant diseased; 4 = Petioles collapse and 80% of the plant diseased; 5 = Entire plant dead.

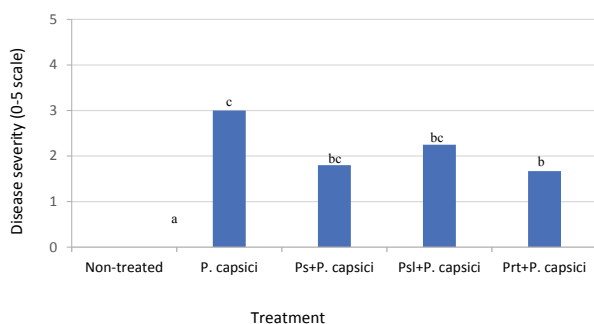


Figure 3: Effect of bacterial endophytes on phytophthora blight severity on bell pepper (California Wonder). Values are means of six replicates. Means followed by the same letter are not significantly different at 5% level by Fisher's LSD.

Treat-ment	Chlo-rophyll content	Root length (cm)	Shoot length (cm)	Plant fresh weight (g)	Plant dry weight (g)	Number of fruits/plant	Fruit weight (g)/plant
Control	7.24a*	32.28a	30.45a	63.18a	7.78a	0.75a	7.48a
Ps	9.41a	30.92a	40.98b	108.58ab	8.50a	2.20b	48.14b
Psl	5.98a	28.33a	31.00a	90.08ab	9.03a	2.00b	22.55ab
Prt	8.96a	30.73a	32.02a	110.90b	8.75a	2.00b	41.72b

*Means followed by the same letter are not significantly different at 5% level by Fisher's LSD

Table 3: Effect of bacterial endophytes on the growth and yield of bell pepper (*Capsicum annum* 'California Wonder') under greenhouse conditions.

Variables	Bacterial isolates						
	B17B	E	IMC8	Y	Ps	Psl	Prt
B17B		-		+		-	nc
E	-		-	-	nc	-	nc
IMC8		-		nc	-	-	-
Y	+	-	nc		-	-	-
Ps		nc	-	-		-	nc
Psl	-	-	-	-	-		-
Prt	nc	nc	-	-	nc	-	

+: Compatible; -: Not compatible; nc: Not clear

Table 4: Compatibility between bacterial isolates as determined by modified agar diffusion test.

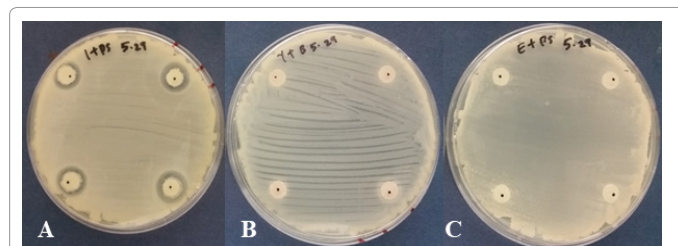


Figure 4: Compatibility tests between combinations of two bacterial endophytes in modified agar diffusion plates. (A) incompatible interaction with clear zone of inhibition, (B) compatible interaction in which two organisms grew together and mixed, (C) unclear interaction with no inhibition zone and not growing together.

weight, fruit yield in number and fruit weight as shown in Table 3. All three *Bacillus* isolates promoted plant growth in shoot height, fruit number and fruit weight with Ps and Prt being slightly better than Psl (Table 3). However, the chlorophyll contents, root length, and plant dry weight of all treated and non-treated plants were not significantly different at $P \leq 0.05$ (Table 3).

Compatibility between bacterial endophytes

Dual cultures between isolates B17B and Y grew well together without inhibiting each other's growth; they were considered compatible. Combinations of isolates B17B and Prt, E and Ps, E and Prt, IMC8 and Y, and Ps and Prt did not show any clear zone of inhibition between organisms and did not mix with each other in their growth. It was unclear whether these organisms were compatible with each other or not (Table 4). Other combinations of isolates such as Prt and B17B, Ps and E, Y and IMC8, E and Ps had a clear zone of inhibition (Figure 4) and were considered incompatible (Table 4).

Sensitivity of Ps, Psl, Prt and *P. capsici* isolate to mfenoxam

There was no significant difference in growth of the three *Bacillus* isolates in media embedded with 10 or 100 $\mu\text{g ml}^{-1}$ of mfenoxam compared with the non-embedded medium. Growth on the media amended with 100 $\mu\text{g ml}^{-1}$ was 40% greater than on non-amended

Treatment ($\mu\text{g ml}^{-1}$)	BCA concentrations at OD600			<i>Phytophthora capsici</i> isolate	
	Ps	Psl	Prt	Mycelial growth (cm)	% Growth Inhibition
0	0.83b*	0.82bc	0.72ab	8.44a	0.00a
5	NT	NT	NT	1.37b	83.78b
10	0.72a	0.76b	0.59b	1.28c	84.89c
100	0.79b	0.86c	0.78a	1.08d	87.19d
1000	0.67a	0.64a	0.65b	NT	NT

*Means followed by the same letter are not significantly different at $P \leq 0.05$; BCA, biological control agents; OD600, optical density measured at 600 nm; NT, not tested

Table 5: Sensitivity of *Bacillus* isolates Ps, Psl and Prt and *Phytophthora capsici* to different levels of mefenoxam (Ridomil Gold SL, 480 mg a.i ml^{-1}).

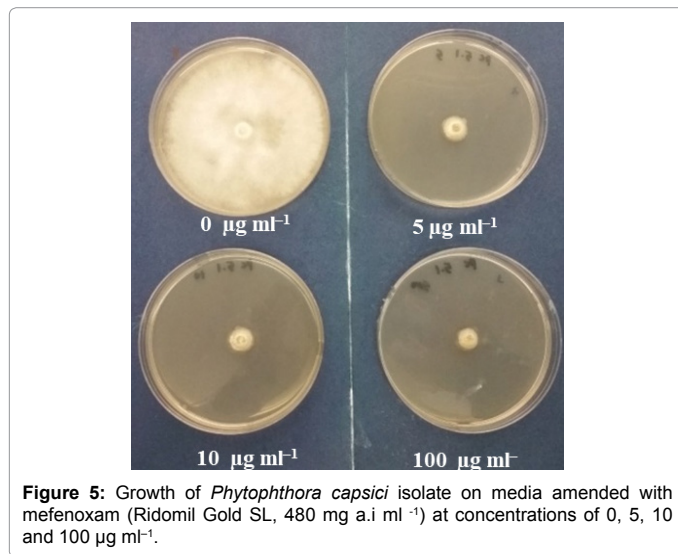


Figure 5: Growth of *Phytophthora capsici* isolate on media amended with mefenoxam (Ridomil Gold SL, 480 mg a.i ml^{-1}) at concentrations of 0, 5, 10 and 100 $\mu\text{g ml}^{-1}$.

medium. Although growth was slightly reduced at 1000 $\mu\text{g ml}^{-1}$ mefenoxam, the difference was less than 40% of the growth on the non-amended medium (Table 5). Hence, *Bacillus* isolates Ps, Psl, Prt they were all characterized resistant to mefenoxam [40].

Phytophthora capsici mycelial growth on the medium amended with 5 $\mu\text{g ml}^{-1}$ of mefenoxam was less than 40% of its growth on the non-amended medium. Mycelial growth decreased significantly with an increase in mefenoxam concentration compared with the non-amended control. Hence the *P. capsici* isolate was characterized to be sensitive to mefenoxam (Table 5 and Figure 5).

Discussion

Endophytes share an intimate life-long relationship with their host plants. This presents an excellent opportunity for their potential utilization as biological control agents [30]. Their ability to colonize the same ecological niche as plant pathogens [32] make them particularly attractive for use as biological control agents against plant diseases. While some endophytes can colonize a broad range of hosts, some are more specific in their host range [30]. Evaluations of several bacterial endophytes isolated from papaya (*Carica papaya*), snap bean (*Phaseolus vulgaris*) and flowering dogwood (*Cornus florida*) for biological control potential against phytophthora blight in bell pepper showed that all isolates were potential biocontrol agents and significantly inhibited mycelial growth of *P. capsici* on LB and NA media. The degree of inhibition observed varied among isolates and growth media. The growth differences could be due to the nutrient composition of the growth media [41,42]. A clear zone of inhibition

on *P. capsici* mycelial growth caused by all bacterial isolates suggests the production of secondary metabolites that antagonized growth of *P. capsici* in culture. Chung et al. [16] reported that *Bacillus subtilis* isolate ME488 displayed broad-spectrum antibiotic activity *in-vitro*; it suppressed 39 different plant pathogens belonging to the genera *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, and *Sclerotinia*.

Reports on *Bacillus* spp. biological control activity against plant pathogens have shown that antibiosis, competition, plant growth promotion and induced systemic resistance were involved [24]. Results obtained from the dual culture assays only show direct antagonism that may be due to the production of antibiotics and/or lytic enzymes. Bacteria in the *Enterobacter* and *Serratia* genera are also plant growth-promoting rhizobacteria known to suppress pathogens by producing antimicrobial compounds and competing for space and nutrients [24]. The most effective BCA usually inhibit plant pathogens using multiple mechanisms of action including direct contact, competition for space and nutrients and/or production of secondary metabolites [19]. Results of the *in vitro* studies suggest that the identification of secondary metabolites as a mechanism of action could be enhanced by the inclusion of different growth media.

The use of *Bacillus* spp. as biocontrol agents against soil-borne plant pathogens is advantageous because they form endospores, easily colonize plant roots, produce broad-spectrum antibiotics, and promote plant growth [43]. The ability of *Bacillus* spp to form endospores make them more resistant to harsh environmental conditions, more tolerant to chemical fungicides and easier to formulate into commercial bio-fungicides with longer viability and shelf-life in comparison to other BCAs that are non-endospore formers [14]. Some *Bacillus*-based bio-pesticides such Kodiak, Companion, Serenade, and Rhapsody are currently being sold and used against many important plant pathogens [44,45]. Although *Enterobacter* and *Serratia* have also been shown to be effective against many soil-borne pathogens [26,46], our *in vivo* studies focused on only three *Bacillus* isolates Prt, Ps and Psl that displayed superior effect in suppressing *P. capsici* growth by more than 70% on both LB and NA growth media compared to the other four isolates tested (Table 2). The selection of these three isolates was also based on previous bioactivity displayed *in vitro* and *in vivo* studies on charcoal rot of snap beans [Jacqueline Joshua, unpublished].

Results from our greenhouse studies with these three *Bacillus* isolates Prt, Ps and Psl confirmed our *in vitro* results. Seed treatment with Prt, Ps, and Psl reduced disease severities in bell peppers compared to the non-treated control (Figure 3). While all three treatments suppressed disease severity in greenhouse studies, only Prt was significantly different from the non-treated control; the Ps and Psl treated plants were not significantly different from Prt-treated plants, but they were also not significantly different from the non-treated control plants at $P \leq 0.05$ (Figure 3). The application of the BCA was done as a onetime seed treatment 1 h before planting. Although the BCA were re-isolated from the stem tissue of treated plants, it is reasonable to presume that more applications would have provided quantitatively more BCA and been more effective in suppressing disease development. Thus, more studies on the mode and frequency of BCA applications are needed.

In addition to suppressing disease severity, this study showed that all three *Bacillus* isolates significantly increased plant growth in shoot height, and fruit number and fruit weight with Ps and Prt being slightly better than Psl (Table 3). Chlorophyll contents, root length, and plant dry weight of treated and non-treated plants were not significantly different at $P \leq 0.05$ (Table 3). Fertilizer application every two weeks

may have provided abundant nutrients and affected the display of growth benefit from these BCAs. However, increasing the frequency of BCA application would likely improve the growth promoting attributes. Other studies have reported that *B. subtilis* isolate ME488 significantly suppressed phytophthora blight of red pepper and plants performed better than those treated with fungicides [16]. Similar results include a reduction in phytophthora blight severity in pepper [44] and a reduction in early blight incidence and increased fruit yield in tomato attributed to inoculations with *B. subtilis* isolates [19]. Many effective BCAs control plant pathogens by stimulating plant defense mechanisms, inducing systemic resistance and/ or plant growth promotion [24]. Increased plant vigor resulting from BCA treatment may have been partly responsible for the reduced severity of Phytophthora blight.

Results from *in vitro* studies are not always confirmed. Biocontrol agents that show low *in vitro* inhibition have been reported to significantly reduce the incidence of diseases caused by the same pathogens in greenhouse and field trials. Someya et al. [47] observed that the isolate *Serratia marcescens* B2 showed negligible inhibition of *Fusarium oxysporum* f. sp. *lycopersici* in *in vitro* studies, but reduced the incidence of Fusarium wilt of cyclamen plants by 50% when applied as soil treatment suggesting that the BCA controls the disease by indirect antagonism. Similarly, Melnick et al. [32] reported that *Bacillus cereus* isolate BT8 had no antagonism against *P. capsici* *in vitro*, but suppressed lesion development on cocoa (*Theobroma cacao* L.) leaves without colonizing them, strongly suggesting induced systemic resistance. Our results from *in vitro* studies were confirmed by our greenhouse study where the use of seed treatment with BCA suppressed phytophthora blight severity and improved plant growth. Secondary metabolites and other mechanisms for the improvement in plant growth could have been involved. Additional studies are required to verify mechanisms involved in biocontrol activity from these organisms.

Additive and synergistic effects resulting from a combination of two or more bacterial antagonists have been reported [19,48]; such enhanced biocontrol activity is likely due to a combination of various mechanisms of control utilized by the microbial antagonists [16,48]. However, the antagonists are required to be compatible to ensure effective and consistent control [19]. Compatibility between various bacterial isolates was evaluated and the results showed that only B17B and Y were clearly compatible. This observation confirms previous results that demonstrated that B17B and IMC8 were compatible [Emily Rotich, unpublished]. However, *in vivo* studies for all isolates selected and their mechanisms of action will guide us to determine the best combinations of isolates for synergistic efficacy in biological control.

The phenylamide fungicide metalaxyl has been used for controlling oomycete diseases in several crop species since it was introduced in the 1970s. Excessive use of metalaxyl has led to the rise of metalaxyl-resistant strains of pathogenic Phytophthora including *P. capsici* and *P. infestans*. Consequently, it was replaced by its more active enantiomer, mefenoxam which has provided effective systemic control of various Phytophthora diseases. Intensive use of mefenoxam has also resulted in the emergence of mefenoxam-resistant strains of *P. capsici* which is now being found in many fields in the United States [40]. Groves and Ristaino [49] observed that the commercial fungicides Ridomil 2E (metalaxyl), Ridomil Gold EC (mefenoxam) at Maneb, Manzate (Mancozeb), Curzate (cymoxanil + mancozeb), and Acrobat MZ (dimethomorph + mancozeb) induced *in vitro* oospore formation in *P. infestans* and changes in mating type expression after only 2 to 4 weeks of exposure. In this study, the sensitivity to mefenoxam of the *P. capsici* isolate used showed that the isolate was sensitive to the fungicide since its growth in 5 ppm was less than 40% of its growth on the non-amended media [40] (Figure 5).

Sensitivity of *Bacillus* isolates Ps, Psl and Prt to mefenoxam at 0 ppm, 10 ppm, 100 ppm, and 1000 ppm showed that all isolates tested were tolerant to mefenoxam even at concentrations higher than the manufacturer's recommended rates. Combining biological control agents with fungicides for plant disease control should reduce the rate of fungicide needed for effective disease management [22]. However, reducing the rate of mefenoxam could result in the development of new pathogenic strains of *P. capsici*. The potential of including BCAs with mefenoxam can be in form of rotations with mefenoxam to reduce the frequency of fungicide applications for a more environmentally friendly, long lasting, consistent, and effective control of *P. capsici*. Similar results on BCA have been reported in other studies [50-54].

Conclusion

Results presented here indicate that isolates tested in this study have biological control potential against phytophthora blight and may be useful in strategies integrating biological control agents with chemical control of Phytophthora diseases.

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