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# Suppression of pecan and peach pathogens on different substrates using *Xenorhabdus bovienii* and *Photorhabdus luminescens*

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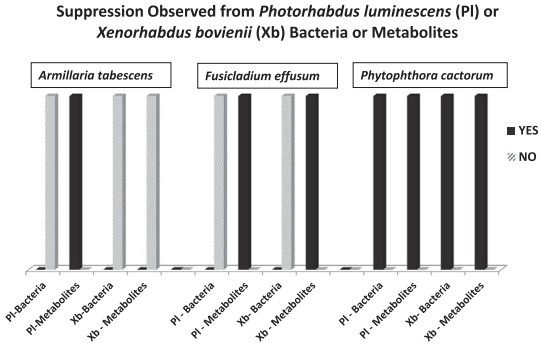
## HIGHLIGHTS

- *Xenorhabdus* and *Photorhabdus* bacteria or metabolites suppressed phytopathogens.
- Toxicity was observed on leaf surfaces, pecan terminals, and in soil.
- This is the first report of *Photorhabdus* or *Xenorhabdus* toxicity to *Armillaria* spp.
- Using metabolites or bacterial broth for suppression of crop diseases is promising.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Prior research indicated the ability of concentrated metabolites from *Xenorhabdus* spp. and *Photorhabdus* spp. to suppress a variety of peach and pecan diseases *in vitro*, and on detached pecan leaves or terminals. In the current study, our objectives were to (1) determine if bacterial broths (in addition to concentrated metabolites tested previously) have suppressive ability and (2) determine if metabolites or bacterial broths are active in a soil medium. In laboratory studies, two pathogens of pecan (*Fusicladium effusum* and *Phytophthora cactorum*) and one peach pathogen (*Armillaria tabescens*) were tested for susceptibility to *Xenorhabdus bovienii* (SN) and *Photorhabdus luminescens* (VS) bacterial broths or concentrated metabolites on three different substrates. Treatments were applied to lesions of *F. effusum* on terminals to ascertain any suppressive effect on sporulation, to *A. tabescens* in soil to determine effect on survival of mycelia, and to lesions caused by *P. cactorum* on pecan leaf surfaces to assess any reduction in lesion development. Acetone (the metabolite solvent), un-inoculated media (tryptic soy broth) and water were included as controls. The *X. bovienii* metabolite treatment was as efficacious as a commercial fungicide (fenbuconazole) in reducing sporulation of *F. effusum* on pecan terminals. The *P. luminescens* metabolite treatment also caused reduced sporulation relative to water and acetone controls but bacterial broths had no effect. In contrast, all bacterial broth and metabolite treatments suppressed lesion growth caused by *P. cactorum* (measured on detached leaves maintained on agar). However, in soil, only the *P. luminescens* metabolite treatment was suppressive to *A. tabescens* (this is the first report of *Photorhabdus* or *Xenorhabdus* toxicity to *Armillaria* spp.). This study provides a basis for further research on the use of *Xenorhabdus* and *Photorhabdus* metabolites or bacterial broth for suppression of pecan and peach diseases.

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## 1. Introduction

Fungal and oomycete induced diseases can severely limit the commercial productivity of pecans, peaches, and other fruit and nut trees in the Southeastern US (Timmer and Duncan, 1999; Teviotdale et al., 2002; Horton and Johnson, 2005; Wells et al., 2007; Horton et al., 2013; Brenneman et al., 2014). Among the economically important pathogens in the Southeastern US are *Fusicladium effusum* (Winter) and *Phytophthora cactorum* (Lebert & Cohn) on pecan, and *Armillaria* spp. on peach (Teviotdale et al., 2002; Horton and Johnson, 2005; Wells et al., 2007; Brenneman et al., 2014). Pathogens within these genera also impact a substantially larger geographic area than the Southeastern US and affect other economically important crops (Coetzee et al., 2000; Cooke et al., 2000; Alexander and Stewart, 2001; González-Domínguez et al., 2013). Curative control measures for *Armillaria* spp. in Southeastern peaches are lacking. Therefore, safe and effective means of control must be developed. Currently, *F. effusum* and *P. cactorum* are controlled by chemical fungicides (Horton and Johnson, 2005; Brenneman et al., 2014). However, due to environmental concerns (National Research Council, 1989) and concerns over resistance development (National Research Council, 1989; Reynolds et al., 1997; Reeleder et al., 2007; Seyran et al., 2010), research toward development of alternative control methods is warranted.

Bacteria and bacterial metabolites that have antimicrobial properties have been investigated for suppression of plant diseases (Emmert and Handelsman, 1999; Shoda, 2000). Indeed, a number of investigations have explored the potential to control pathogens that infect peach and pecan using bacteria and or their metabolites (Pusey et al., 1988; Alexander and Stewart, 2001; Larena et al., 2005; Altindag et al., 2006; Baumgartner and Warnock, 2006). Yet none of these approaches have been adopted for commercial production in the Southeastern US (Horton et al., 2013; Brenneman et al., 2014). Thus, additional research on bacteria and bacterial metabolites for suppression of pecan and peach diseases is needed. Toward that end, research on the potential application of metabolites derived from *Xenorhabdus* spp. and *Photorhabdus* spp. bacteria has been initiated (Shapiro-Ilan et al., 2009,Shapiro-Ilan and Reilly; 2013; Bock et al., 2014).

*Xenorhabdus* spp. and *Photorhabdus* spp. produce antibiotic compounds which are known to have suppressive effects on a variety of fungi including important plant pathogens (Akhurst, 1982; McInerney et al., 1991; Chen et al., 1994; Li et al., 1995; Webster et al., 1995; Ng and Webster, 1997; Isaacson and Webster, 2002; Webster, et al. 2002). These bacteria are mutualistic symbionts of entomopathogenic nematodes (*Xenorhabdus* spp. are associated with *Steinernema* spp. and *Photorhabdus* spp. are associated with *Heterorhabditis* spp.), which are used as biological control agents of insects (Kaya and Gaugler, 1993; Grewal et al., 2005; Shapiro-Ilan et al., 2014). In nature, the bacteria exist only in the intestine of their nematode symbionts or in the insect hosts that nematodes infect; the bacteria require the protection of the nematode to survive in the external environment (Griffin et al., 2005; Lewis and Clarke, 2012). The bacteria can, however, be cultured *in vitro* on solid media or in liquid fermentation (Shapiro-Ilan and Gaugler, 2002; Ehlers and Shapiro-Ilan, 2005).

In a previous laboratory study, zone of inhibition assays conducted on agar media indicated that concentrated metabolites from several strains or species of *Xenorhabdus* and *Photorhabdus* are toxic to various pecan and peach diseases including *Glomerella cingulata* (Stoneman), *Phomopsis* spp., *P. cactorum*, and *F. effusum* and *Monilinia fructicola* (Winter) (Shapiro-Ilan et al., 2009). Metabolites from two bacteria isolates, *Xenorhabdus bovienii* (SN strains) and *Photorhabdus luminescens* (VS strain) were determined to have superior toxicity relative to others tested (Shapiro-Ilan et al., 2009). Additionally, metabolites suppressed growth of *P. cactorum* on detached pecan leaves, and sporulation of *F. effusum* on pecan terminals.

The results of Shapiro-Ilan et al. (2009) study demonstrated the potential for using *Xenorhabdus* and *Photorhabdus* metabolites as suppressive agents against phytopathogens of peach and pecan. However, the study did not determine whether concentrated metabolites are required for suppression or if *Xenorhabdus* and *Photorhabdus* bacterial broths may also be toxic. Conceivably, due to costs and/or regulatory issues, application of bacteria or bacterial broths may be more feasible in some circumstances relative to application of concentrated metabolites. Furthermore, the previous study (Shapiro-Ilan et al., 2009) did not determine potential toxicity in a soil medium. Suppression in soil could be highly beneficial because many phytopathogens occupy the soil environment for part or all of their life-cycles (Weller et al., 2002; Baumgartner and Warnock, 2006). This study was devised to address these knowledge gaps. Thus, the objectives were to (1) determine if bacterial broths (in addition to concentrated metabolites tested previously) have suppressive ability and (2) determine if metabolites or bacterial broths are active in a soil medium. The potency of *X. bovienii* (SN) and *P. luminescens* (VS) bacterial broths or concentrated metabolites was determined when applied to lesions of *F. effusum,* on terminals, *Armillaria tabescens* (Scopoli) in soil, and *P. cactorum* on pecan leaf surfaces.

## 2. Materials and methods

### 2.1. Bacteria and pathogen cultures and extraction of metabolites

Cultures of *P. cactorum* were grown on potato dextrose agar (PDA, (Becton Dickinson, Sparks, MD) in Petri dishes in an incubator maintained at 25 °C. *A. tabescens* was grown on peach bark medium (per liter: 25 g peach bark [Lovell variety], 30 g malt extract [Sigma Aldrich, St Louis, MO], and 25 g Bacto agar [Becton Dickinson & Co., Sparks, MD], 0.1 g streptomycin [Sigma Aldrich, St Louis, MO], pH adjusted to 5.5.

The bacteria *P. luminescens* (VS) and *X. bovienii* (SN) were isolated in parallel from their nematode symbionts *Heterorhabditis bacteriophora* Poinar (VS strain) and *Steinernema feltiae* (Filipjev) (SN strain), respectfully. The nematodes were cultured in last instar *Galleria mellonella* (L.) according to procedures described by Kaya and Stock (1997). Bacterial colonies were established on nutrient agar by streaking hemolymph from insects previously infected with nematodes. *Photorhabdus* spp. and *Xenorhabdus* spp. occur as two phase variants (primary and secondary), yet for the most part it is only the primary phase that produces antibiotics (Akhurst, 1982; Forst and Clarke, 2002). Thus, it was in our interest to maintain bacteria in the primary form. Selective media (Tergitol-7-agar) was used to indicate primary variant characteristics during bacterial isolation and culturing (Kaya and Stock, 1997; Forst and Clarke, 2002). For bacterial broth treatments (see below), fresh cultures were generated by adding an aliquot of bacteria to 50 ml of TSY [Tryptic Soy Broth (Difco, Detroit, MI) + 0.5% yeast extract (Sigma, St. Louis, MO)] in a 250 Erlenmeyer flask that was placed on a rotary incubator shaker (Innova 4230, New Brunswick Scientific, Edison, NJ) at 25 °C and 130 rpm for 18–24 h. The treatment cultures were stored at 4 °C for <24 h before use and standardized at approximately $4 \times 10^8$ cells per ml. A different batch of bacteria was used in each trial.

Soluble organic metabolites were isolated from the bacteria based on procedures described by Ng and Webster (1997). Briefly, cultures generated in 50 ml TSY (as described above) were then transferred to 900 ml TSY in 2-L flasks and placed on a rotary shaker at 25 °C for 96 h. The cells and broth were centrifuged at 10,000 rpm (RCF = 14,224 g) for 20 min. Supernatants were

extracted three times with ethyl acetate (Fisher Scientific, Fair Lawn, NJ), organic fractions were dried with anhydrous ammonium sulfate (Sigma, St. Louis, MO), concentrated on a rotary evaporator, and dissolved in acetone (100 mg/ml) (Sigma, St. Louis, MO). The solutions were stored at 4 °C until use.

### 2.2. Suppression of F. effusum on pecan terminals

Methods for measuring suppression of *F. effusum* were based on those described by Shapiro-Ilan et al. (2009). Terminals exhibiting *F. effusum* lesions were collected in the early spring of 2009 from pecan orchards (variety = Wichita) from USDA-ARS orchards in Byron, GA. The terminals were selected for uniformity in diameter, cut into 5 cm segments, and placed individually into 1.5 × 10 cm test tubes. The terminals were exposed to the following four treatments: undiluted bacterial broth or metabolites derived from either *P. luminescens* (VS) or *X. bovienii* (SN). Acetone (the metabolite solvent), non-inoculated TSY, and water were included as negative controls. A standard chemical fungicide used for control of *F. effusum* (Brenneman et al., 2014), fenbuconazole (Enable 2F™, 240 g/L active ingredient, flowable, Rohm and Haas, Philadelphia PA), was used as a positive control and applied at the standard field rate. Treatments were applied (at approximately 0.75 ml per terminal) using an airbrush sprayer (Badger 175-9, Tower Hobbies, Champagne, IL). The terminals were incubated at 25 °C for 72 h at 100% RH, and then exposed to sonification for 30 min in 6 ml of 1% Tween 20 solution (Fischer Scientific, Fair Lawn, NJ) and the number of spores released was counted and spores per lesion and terminal were calculated number of spores released per lesion was counted on a hemocytometer. There were 10 replicate terminals for each treatment (with 43–90 lesions per terminal) and the experiment was repeated once (two separate trials in time).

### 2.3. Suppression of P. cactorum on pecan leaves

Suppression of *P. cactorum* on detached pecan leaves was addressed based on procedures described by Ng and Webster (1997) and Shapiro-Ilan et al. (2009). Treatments included 12.5% and 25% dilutions of the two metabolite solutions (*P. luminescens* and *X. bovienii*), the two bacterial broths (undiluted) and the same negative controls as described above for the *F. effusum* experiment; dilutions of metabolite solutions were made with distilled water. Agar plugs of *P. cactorum* (approximately 0.5 cm in diameter) were placed fungus-side down on young pecan leaves (½ to ¾ expanded, cultivar = Desirable) that had previously been sprayed with 200 µl of the treatment or control by airbrush and allowed to dry. The leaves were placed on 1% water agar, and incubated at 25 °C. After 4 and 7 days the development of lesions caused by *P. cactorum* infection was assessed. The maximum length of each lesion across 2 perpendicular directions of each lesion was measured (Shapiro-Ilan et al., 2009). Each treatment and the controls were replicated 5 times and the experiment was repeated twice (three trials in time).

### 2.4. Suppression of A. tabescens on agar and in soil

Toxicity of *Photorhabdus* or *Xenorhabdus* treatments applied to *Armillaria* spp. had not previously been tested. Therefore, prior to determining effects in a soil medium, we had to establish susceptibility of the fungus to metabolites. To ascertain susceptibility, an *in vitro* zone of inhibition assay was conducted on agar plates (Shapiro-Ilan et al., 2009). Briefly, *A. tabescens* suspensions were prepared by mixing mycelia produced by the fungus on peach bark agar in 20 ml sterile distilled water (sdw). Using an airbrush sprayer, approximately 300 µl of the *A. tabescens* suspensions was sprayed on fresh peach bark agar surfaces, and a filter paper disc (1 cm diam) with bacterial metabolites added (20 µl) was placed in the center. Control plates received filter paper disks with only the acetone or water (20 µl). After 96 h the area of the inhibition zone was calculated based on the average of two diameters measured in two perpendicular axes (fungus growing under the disc was included in the measurement). A separate assay was conducted for each metabolite (*X. bovienii* and *P. luminescens*). The treatments and controls were each replicated three times, and in the case of the *P. luminescens* assay the experiment was repeated once in time.

To determine suppressive ability in a soil medium, experimental arenas consisted of well plates (12 cm × 8.3 cm with 2.5 cm diam. wells) containing 2.5 g dry, autoclaved soil. The soil was a loamy sand (Norfolk loamy sand [Kaolinitic, Thermic Typic Kandiudult]) with the percentage sand:silt:clay of 84:10:6, pH 6.1, and organic matter of 2.8% by weight. Approximately 100 mg of *A. tabescens* (scraped from culture plates and macerated with a tissue grinder [Qiagen Tissue Lyser II, Boston Laboratory Equipment, Woburn, MA]) was added to each well in 175 µl of sdw and mixed thoroughly. Treatments and controls were the same as described above for the *F. effusum* experiment (except a chemical fungicide was not included). Approximately 225 µl of undiluted bacterial broth treatments, un-inoculated TSY, or water-only (sdw) were applied to each well, whereas 50 µl of the metabolites or acetone control were mixed with 175 µl sdw and then applied to soil. The total volume of liquid added to each plate was equivalent to the soil's field capacity. The contents of each well were mixed and plates were covered with Parafilm® and incubated at 25 °C. After 72 h, soil from each well was added to 9 ml sdw and the suspensions were vortexed for 1 min. Approximately, 0.1 ml of each suspension was pipetted onto a 90 mm Petri plate containing selective medium (Kuhlman and Hendrix, 1962) and spread with a bent glass rod. Plates were incubated at 25 °C for 3 d (trial 1) or 5 d (trial 2) at which time the number of colony forming units (CFUs) per plate was determined. There were four replicate wells for each treatment and the entire experiment was repeated once in time (two trials).

### 2.5. Statistical analyses

Treatment effects were determined using ANOVA (SAS, 2002). Numerical data from all experiments were square-root transformed prior to analysis (Southwood, 1978; Steel and Torrie, 1980); non-transformed means are presented in the figures. Data from repeated experiments (trials) were pooled and trial was considered as a block effect. If a significant treatment effect was detected in the ANOVA, then the treatment differences were further elucidated through the Student–Newman-Keuls' test (SAS, 2002). The alpha level for all statistical tests was 0.05.

## 3. Results

Relative to the negative controls, *F. effusum* sporulation was suppressed by the *X. bovienii* metabolite treatment, and the level of suppression was similar to that achieved by the chemical fungicide standard, fenbuconazole ($F = 8.17$; df = 7, 151; $P < 0.0001$) (Fig. 1). The *P. luminescens* metabolite treatment also exhibited some suppressive ability as the number of spores produced was lower than the water or acetone controls though not different from non-inoculated TSY broth (Fig. 1). The bacterial broth treatments (with *X. bovienii* or *P. luminescens*) did not suppress sporulation relative to the controls (Fig. 1).

All bacterial broth and metabolite treatments suppressed the development of lesions caused by *P. cactorum* on detached pecan leaves at 4 d ($F = 10.49$; df = 8, 123; $P < 0.0001$) and 7 d ($F = 9.47$;

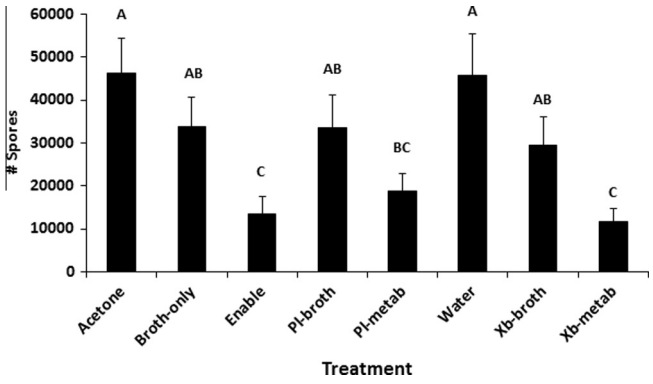


**Fig. 1.** Number of surviving spores of *Fusicladium effusum* following treatments of *Xenorhabdus bovienii* (Xb) and *Photorhabdus luminescens* (Pl) bacterial broths or concentrated metabolites applied to pecan terminals. The fungicide Enable™ (fenbuconazole) was applied as a positive control and acetone (the metabolite solvent), non-inoculated media broth, and water were applied as negative controls. Different letters above bars indicate statistical significance (Student–Newman-Keuls' test, $\alpha = 0.05$).

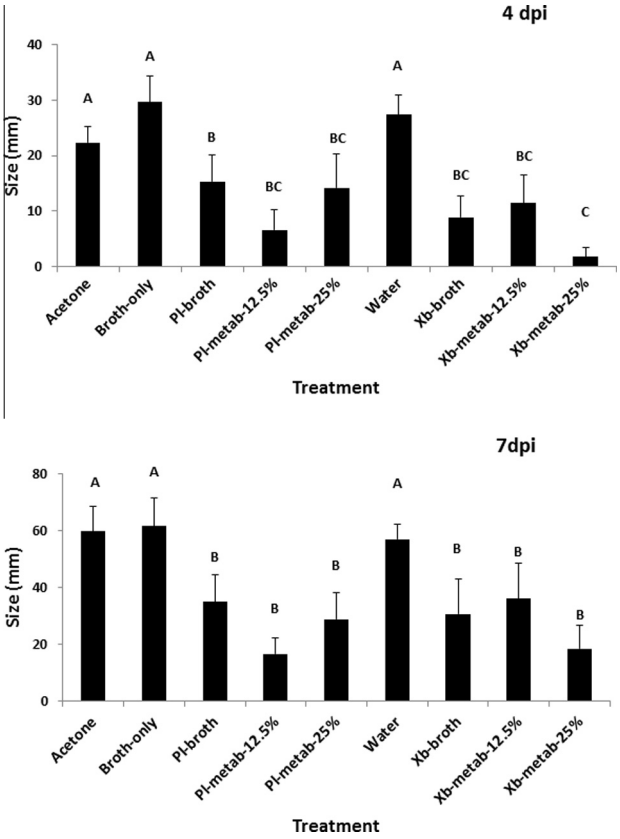


**Fig. 2.** Size of *Phytophthora cactorum* lesions on detached pecan leaves 4 and 7 d post-inoculation (dpi) treatments of *Xenorhabdus bovienii* (Xb) and *Photorhabdus luminescens* (Pl) bacterial broths or concentrated metabolites. Acetone (the metabolite solvent), non-inoculated media broth, and water were applied as controls. Different letters above bars indicate statistical significance (Student–Newman-Keuls' test, $\alpha = 0.05$).

df = 8, 124; $P < 0.0001$) post-inoculation (Fig. 2). At 4-d-post-inoculation, no differences among metabolite or bacterial broth treatments were detected except the *X. bovienii* metabolite treatment (25%) resulted in smaller lesions and thus greater suppression compared with the *P. luminescens* broth (Fig 2). At 7-d-post-inoculation, no difference among metabolite or bacterial broth

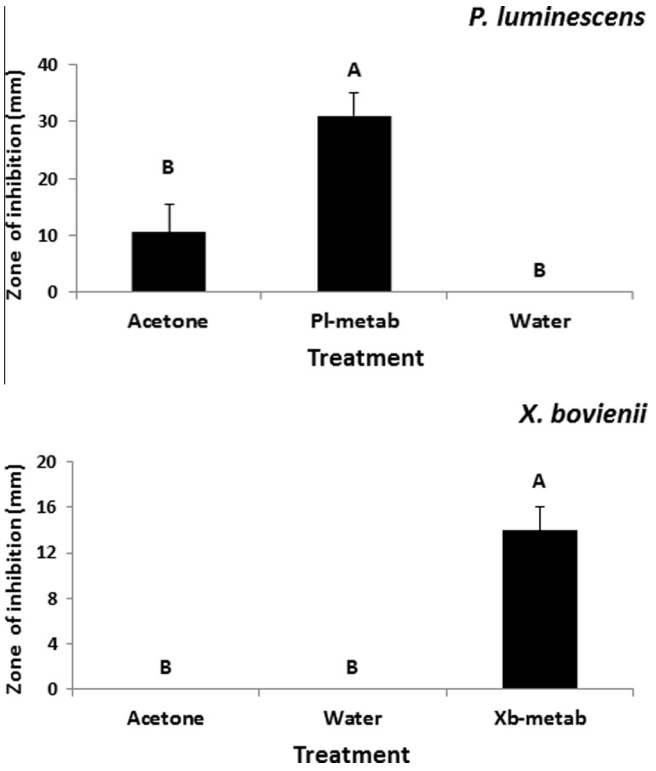


**Fig. 3.** Mean zone of inhibition on agar plates caused by *Xenorhabdus bovienii* (Xb) or *Photorhabdus luminescens* (Pl) metabolites applied to lawns of *Armillaria tabescens*. Acetone (the metabolite solvent) and water were applied as controls. Different letters above bars indicate statistical significance (Student–Newman-Keuls' test, $\alpha = 0.05$).

treatments were detected and all treatments were different from controls (which were statistically similar to each other) (Fig. 2).

Zone of inhibition tests conducted on agar plates indicated that both metabolite treatments were toxic to *A. tabescens* ($F = 19.23$; df = 2, 15; $P < 0.0001$ for the *P. luminescens* metabolite assay, and $F = 48.0$; df = 2, 6; $P = 0.0002$ for the *X. bovienii* assay) (Fig. 3). Subsequently, in soil assays, the *P. luminescens* metabolite treatment suppressed *A. tabescens*; the viability of the mycelial fragments as measured by CFUs on selective media was reduced relative to the controls ($F = 7.22$; df = 6, 48; $P < 0.0001$) (Fig. 4). In contrast, the *X. bovienii* metabolite and bacterial broth treatments did not exhibit a suppressive effect on mycelial fragments of *A. tabescens* in soil (Fig. 4).

## 4. Discussion

A number of studies have demonstrated the ability of bacteria or their byproducts to control plant diseases (Emmert and Handelsman, 1999; Shoda, 2000). Our results indicate that *P. luminescens* and *X. bovienii* bacteria and/or their metabolites are suppressive to three important pathogens of peach and pecan, and that the activity of these metabolites was observed on three different substrates including soil (as well as disease lesions on terminals and leaf surfaces, as shown previously). The relative toxicity of treatments varied depending on the target fungal pathogen; *X. bovienii* exhibited higher toxicity to *F. effusum* on pecan terminals whereas *P. luminescens* was superior against the peach pathogen *A. tabescens* in soil. Substrate may also be important in determining relative toxicity, e.g., both *P. luminescens* and *X. bovienii* exhibited an inhibitory activity to *A. tabescens* in the zone of inhibition tests conducted on agar, yet only *P. luminescens* was active in soil.

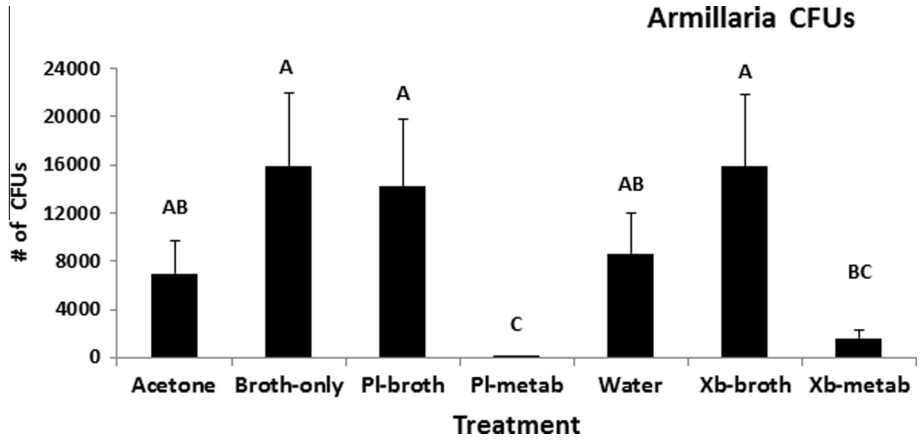


**Fig. 4.** Number of colony forming units (CFUs) after soil infested with mycelial fragments of *Armillaria tabescens* was treated with *Xenorhabdus bovienii* (Xb) and *Photorhabdus luminescens* (Pl) bacterial broths or concentrated metabolites and streaked onto selective media. Acetone (the metabolite solvent), non-inoculated media broth, and water were applied as controls. Different letters above bars indicate statistical significance (Student–Newman-Keuls' test, $\alpha = 0.05$).

In lieu of applying concentrated bacterial metabolites, applications of bacteria and fermentation broth could be advantageous in terms of costs or regulatory issues. However, in our experiments, bacterial broth treatments only suppressed the growth of lesions caused by *P. cactorum* of pecan leaves, while activity to the other pathogens tested (*F. effusum* and *A. tabescens*) was not detected. Conceivably, higher rates of the broth treatment would induce a response. Alternatively, toxicity of active compounds within the broth could be enhanced through media optimization (Wang et al., 2011).

Based on our research and the support of other reports in the literature there appears to be potential for using *P. luminescens* and *X. bovienii* as biocontrol agents for specific diseases in both peach and pecan, and perhaps on other plants. Suppression of *P. cactorum* appears to be particularly promising because all metabolite and broth treatments were highly suppressive, and because prior studies involving other *Phytophthora* spp. indicated suppression by *P. luminescens* and *X. bovienii* in laboratory and field trials (Ng and Webster, 1997; Fang et al., 2011; Böszörményi et al., 2009). Suppression of *F. effusum* sporulation on pecan terminals showed promise especially in that the *X. bovienii* metabolite treatment caused similar reductions compared with a standard chemical fungicide. However, it is not clear if reducing sporulation on pecan terminals early in the season will be a viable tool to suppress *F. effusum* as the crop develops; *F. effusum* is a polycyclic pathogen and thus even minimal spore production early in the season can lead to a rapid epidemic development if the conditions are suitable for repeated infection of the host and sporulation. Thus the ability of bacterial metabolites to directly protect the plant by suppressing infection by *F. effusum* and growth of the pathogen on leaves and fruit also warrants testing. Our results on suppression of *A. tabescens* in soil are also encouraging as our study was the first to report toxicity of *Xenorhabdus* spp. or *Photorhabdus* spp. However, there are thus far limited reports in the literature for the use of bacteria or their metabolites as a tool to control *Armillaria* spp. (Biondi et al., 2004), and due to this dearth of information a considerable amount of research remains to be accomplished before the efficacy or practicality of the approach can be established.

In addition to using concentrated metabolites or bacterial broth treatments, another avenue for pest or disease suppression is the development of bioactive compounds that are responsible for the bacterial toxicity. Various active compounds in *Xenorhabdus* spp. and *Photorhabdus* spp. have been identified (Li et al., 1995; Webster et al., 2002; Böszörményi et al., 2009; Xiao et al., 2012) yet thus far none have been commercialized. Recently, trans-cinnamic acid (TCA) was reported to be a major active compound in *P. luminescens*' suppressive activity against *F. effusum* and thus further research to develop TCA as a potential control agent was suggested (Bock et al., 2014). Additional research on identification and activity of bioactive compounds is warranted. Furthermore, regardless of the type of treatment (bioactive chemicals, metabolites or bacterial treatments) field testing and economic feasibility analysis will be needed as various biotic and abiotic factors outside of the laboratory may reduce potency and longevity of the materials.

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