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# Two Philippine *Photorhabdus luminescens* strains inhibit the in vitro growth of *Lasiodiplodia theobromae, Fusarium oxysporum* f. sp. *lycopersici*, and *Colletotrichum* spp.

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## Abstract

**Background:** Fungal phytopathogens are one of the leading causes of loss in global food production. Chemical fungicides have always been used to control the phytopathogens to mitigate losses. However, it is widely known that this approach is not sustainable. Thus, it is essential to develop alternative control methods, such as the use of biological control agents.

**Results:** This study provided a preliminary data on the efficacy of 2 local *Photorhabdus* strains, associated with *Heterorhabditis indica* BSDS and *H. indica* MAP, against selected post-harvest fungal phytopathogens, *Fusarium oxysporum* f. sp. *lycopersici, Lasiodiplodia theobromae, Colletotrichum musae,* and another *Colletotrichum* sp., by measuring their in vitro inhibitory activity. The *Photorhabdus* strains were isolated from the hemolymph of *Ostrinia furnicalis* infected with *H. indica* BSDS and *H. indica* MAP and grown selectively on NBTA media. Firstly, the bacterial endosymbionts' generic identity was confirmed through colony PCR based on a *Photorhabdus Txp40* toxin-specific marker. Species identity was then elucidated through *16s* marker-assisted GenBank mining as *P. luminescens,* sharing 99.51–99.58% similarity with *P. luminescens* subsp. *akhurstii* (Accession no. AY278643.1). Anti-fungal activity was observed by the bioassay experiments, using cell-free culture filtrates (CFCs), obtained from *P. luminescens* tryptic soy broth suspensions ($OD_{600} = 2.0$) amended in PDA medium (25%v/v) based on percentage growth inhibition. The CFCs of *P. luminescens* BSDS showed a significantly higher suppressive activity against *Colletotrichum musae, Colletotrichum* sp., and *Lasiodiplodia theobromae,* with $93.18 \pm 0.46\%$, $74.15 \pm 0.54\%$, and $60.51 \pm 2.04\%$ growth inhibition, respectively, while the CFC of *P. luminescens* MAP showed a significantly higher suppressive activity against *F. oxysporum* f. sp. *lycopersici* with $21.87 \pm 0.71\%$ growth inhibition.

**Conclusions:** The results strongly showed that these strains of *Photorhabdus* can be promising biological control agents against these fungal phytopathogens. Further extensive research is warranted for the development of these promising biofungicides into a practical, economically viable, and environment-friendly control strategy that can be incorporated into any integrated pest management program.

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## Background

Diseases caused by plant pathogens such as fungi and bacteria are one of the significant contributors to post-harvest losses (Gustafson 2019). Fungal phytopathogens are the most common and destructive causal organisms of plant diseases from pre- to post-harvest stages. Their persistence in different crop production systems is primarily attributed to their complex and varied characteristics (Palm 2001). *Lasiodiplodia, Colletotrichum,* and *Fusarium* are amongst the notorious genera that cause many post-harvest diseases of different crops (Coates and Johnson 1997).

To combat these pathogens, farmers have been dependent on the rapid action and efficiency of chemical fungicides (McGrath 2004). However, it has been well documented that most of these chemical residues are highly toxic and persist for years in the soil (Mahmood et al. 2016). Several studies were done to develop alternative approaches to manage fungal plant pathogens efficiently and sustainably for these reasons. Biological control of fungal plant pathogens, using bacterial antagonists, has been an alternative management strategy against these organisms. Several bacterial species under the genera *Bacillus* (El-Bendary et al. 2016), *Pseudomonas* (Weller 2007) and *Serratia* (Someya et al. 2000) are known to be inhibitory against some significant fungal plant pathogens.

Another group of antagonistic bacteria studied against plant pathogens is the entomopathogenic bacteria (EPB), which comprises species that are used for insect management programs. Recent studies have added *Photorhabdus* and *Xenorhabdus* spp., endosymbiotic bacteria of entomopathogenic nematodes (EPNs), *Steinernema* and *Heterorhabditis,* respectively, on the long list of antagonistic bacteria against many fungal plant pathogens (Fang et al. 2014) such as *Glomerella cingulata*, *Phomopsis* sp., *Phytophthora cactorum*, *Fusicladosporium effusum, Monilinia fructicola* (Shapiro-Ilan et al. 2009), some C*olletorichum* spp. (Bock et al. 2013), *F. carpophylium* and *F. effusum* (Hazir et al. 2016). These bacterial endosymbionts play a significant part in the capability of the EPNs in controlling insect pests (Tofangsazi et al. 2012). Upon entry of the EPNs to their respective hosts, these bacterial endosymbionts are released into the insect hemocoel to provide the EPNs enough nutrition for their growth and development. Part of the EPNs successful invasion is their secondary metabolites, containing either insecticidal toxins or anti-microbial proteins or even both. Some of these proteins are used to prevent the insect cadaver's immediate putrefaction and hamper other microbial organisms from infecting the cadaver (Muangpat et al. 2017).

This research is a pioneering *Photorhabdus* bioefficacy study in the Philippines, designed to determine the in vitro antifungal effect of local *Photorhabdus* strains against postharvest pathogens, *L. theobromae, F. oxysporum* f. sp. *lycopersici*, and *C.* species.

## Methods

For the isolation of the EPB, 200 infective juveniles of the 2 nematode isolates, *Heterorhabditis indica* BSDS and *H. indica* MAP, were used to infect 13-day-old larvae of *Ostrinia furnacalis* (Guenee) (Lepidoptera: Carambidae). After approximately 24 to 48 h. post-infection, the larva's proleg was cut off to extract its hemolymph, which was then streaked onto nutrient agar supplemented with bromothymol blue and triphenyltetrazolium chloride (NBTA). The plates were incubated for 48 to 72 h. until pure green colonies appeared. These were purified further and grown in tryptic soy broth (TSB) and incubated at 28 °C for 24–72 h. in a shaking incubator. Culture stocks were prepared in 50% glycerol solution for long-term storage at − 80 °C.

Colony PCR was performed on Quanta Biotech S96 thermal cycler (Quanta Biotech, Ltd., Surrey, England) using the following programs: i) 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 12 min, 72 °C for 10 min, and 25 °C for 1 min and ii) 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and 72 °C for 5 min, for *Txp40* gene-based *Photorhabdus* (Brown et al. 2005) detection and *16s*-based species identification (Jang et al. 2011), respectively. A 25-µl reaction mix for both markers composed of 12.5 µl of 2 × Taq Master Mix (Vivantis), 1.0 µl of forward and reverse primer (50 pmol), 0.25 µl of $MgCl_2$ (1.5 µM), 7.25 µl nuclease-free water (Vivantis), and 3.0 µl DNA template were used. The amplicons produced were then subjected to gel electrophoresis for 35 min at 100 V using a GelRed® (Biotium)-stained 1% agarose gel submerged in 0.5X TBE buffer. The amplicons were viewed under the AlphaImager Mini System (Protein simple, San Jose California, USA). *16s* amplicons were sent to Apical Scientific Sdn, Bhd. (Malaysia) for Sanger sequencing. Sequences were edited using Bioedit software (Hall 1999) and subjected to megaBLAST for identity mining.

For collecting cell-free culture filtrates (CFC), tubes containing 10 ml TSB were inoculated with a loopful of each bacterial isolate and placed in an incubator shaker under 170 rpm at 28 °C for 48–72 h. The bacterial samples' optical density was measured using a spectrophotometer with a wavelength of 600 nm and adjusted to $OD_{600} = 2.0$. The bacterial samples were then centrifuged at 15,000×g, at 4 °C for 15 min, and the supernatant was collected and passed through a 0.22 µm bacterial filter. The collected CFCs were mixed with PDA (25% v/v) and plated in 60 × 15 mm Petri plates. For the control, uninoculated TSB was added to PDA. With a sterile 0.7 cm cork borer, agar discs were cut off from the 5- to 7-day-old *F. oxysporum* f. sp. *lycopersici*, *L. theobromae,* and 2 *Colletotrichum* spp. cultures, and transferred onto the middle of the PDA plates. The fungal growth's actual diameter was measured for 2–7 days as soon as the control fungus reached the plate's edge. The data were subjected to analysis of variance (ANOVA), followed by Least Significant Difference post hoc test (LSD test), using Rstudio software (V. 4.0.2).

## Results

*Photorhabdus* colonies were verified through the amplification of the *Txp40* toxin gene marker of approximately 1200 bp. The amplified product agrees with the published amplicon sizes specific for this gene. Further characterization of the *Photorhabdus* BSDS and *Photorhabdus* MAP revealed their identity as *P. luminescens* based on *16s* sequences with 99.51 and 99.58% similarity, respectively, to *P. luminescens* subsp. *akhurstii* (Accession No. AY278643.1) in the GenBank-NCBI database. The *16s* sequence data of *Photorhabdus* BSDS and *Photorhabdus* MAP were uploaded to the GenBank-NCBI database with Accession No. MT658664 and MT658665, respectively.

Based on the bioassay experiment, independent analyses showed significant differences in percentage growth inhibition on PDA plates amended with CFCs of both *P. luminescens* BSDS and *P. luminescens* MAP compared to those grown without CFCs. The percentage growth inhibition of *L. theobromae*, *Colletotrichum* sp. and *C. musae* exposed to *P. luminescens* BSDS (60.51 ± 2.04, 74.15 ± 0.54, and 93.18 ± 0.46%, respectively) were significantly higher than those of *P. luminescens* MAP (37.50 ± 2.18, 30.11 ± 1.43, and 88.64 ± 0.66%, respectively). However, a different trend of growth inhibition caused by both CFCs was observed on *F. o.* f. sp. *lycopersici* with *P. luminescens* MAP (21.87 ± 0.71%) being more inhibitory than *P. luminescens* BSDS (9.95 ± 3.36). Relatively, the weakest inhibitory activity from both CFCs was observed against *F.o.* f.sp. *lycopersici*, while the most substantial effect can be seen on *C. musae* (Table 1, Fig. 1).

## Discussion

In this study, CFCs of the bacterial isolates were used for the biological assay instead of the live cells because of the innate phase variation they possess during in vitro culture (Han and Ehlers 2001), which significantly affects anti-microbial metabolite production (Bock et al. 2013). In a similar study, CFCs from phase I variants of *P. luminescens* isolated from *H. megidis* and grown in TSB completely inhibited the growth of the fungal plant pathogens, *Botrytis cinerea*, *Ceratocystis ulmi*, *Ceratocystis dryocoetidis*, *Mucor piriformis*, *Pythium coloratum*, *Pythium ultimum*, and *Trichoderma pseudokingii* (Chen et al. 1994).

**Table 1** Mean percentage growth inhibition of the tested plant pathogenic fungi on potato dextrose agar (PDA) plates treated with CFC of *Photorhabdus luminescens* BSDS and *P. luminescens* MAP

| | **Mean Percentage Growth Inhibition ± S.E** | | | |
|---|---|---|---|---|
| | ***Fusarium oxysporum* f. sp. *Lycopersici* (at 6 dpi)** | ***Lasiodiplodia theobromae* (at 2 dpi)** | ***Colletotrichum* sp. (at 5 dpi)** | ***Colletotrichum musae* (at 4 dpi)** |
| Untreated PDA | – | – | – | – |
| PDA with blank TSB | 0.00[c] | 0.00[c] | 1.14 ± 0.66[c] | 0.00[c] |
| *P. luminescens* BSDS | 9.95 ± 3.36[b] | 60.51 ± 2.04[c] | 74.15 ± 0.54[a] | 93.18 ± 0.46[a] |
| *P. luminescens* MAP | 21.87 ± 0.71[a] | 37.50 ± 2.18[b] | 30.11 ± 1.43[b] | 88.64 ± 0.66[b] |

Separate analysis was done for each bioassay setup using the average of the two trials

Means within the same column are being compared

Means with the same letter are not significantly different at α = 0.05 using the LSD test

Mean percentage fungal growth inhibition is computed using the following formula:

[(Growth diameter in control plates − Growth diameter in treated plates)/Growth diameter in control plates] * 100

CFC, cell-free culture filtrates; dpi, days post infection; TSB, tryptic soy broth

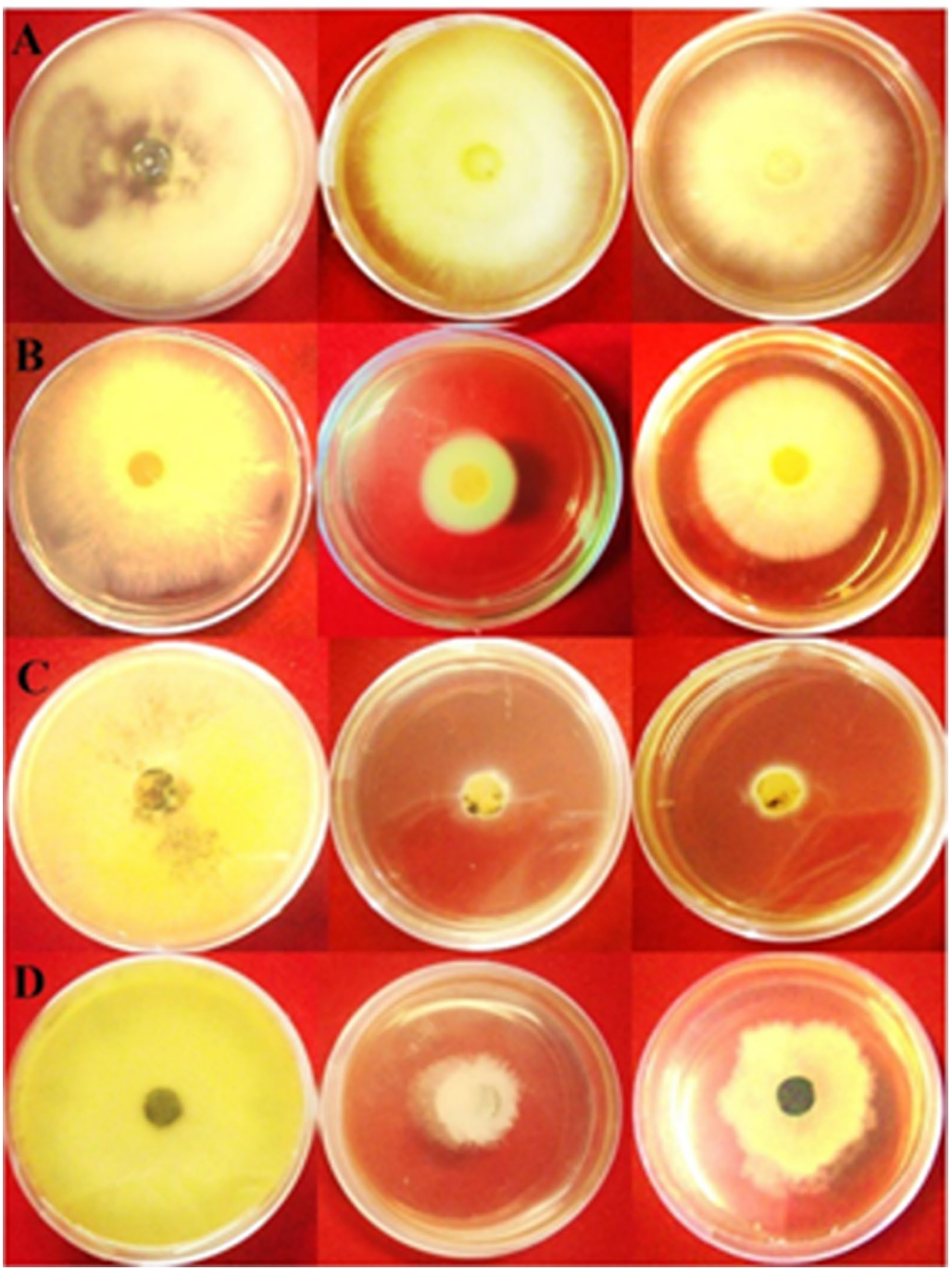


**Fig. 1** Anti-fungal activity of *Photorhadus luminescens* BSDS and *P. luminescens* MAP cell-free culture filtrates against **A** *Fusarium oxysporum* f. sp. *lycopersici* at six days post-infection (dpi); **B** *Colletotrichum* sp. at five dpi; **C** *Colletotrichum musae* at four dpi; and **D** *Lasiodiplodia theobromae* at two dpi on PDA media. The control plates were amended with tryptic soy broth only

Furthermore, the results also indicated that there exists an intraspecific variation in terms of their antifungal effect. Variable mean growth diameter can be caused by the differences in toxicity, forms, and dosages of secondary metabolites found in the CFCs and the fungal phytopathogens' innate sensitivity. Shapiro-Ilan et al.

(2009) also demonstrated the suppressive effects of *Photorhabdus* metabolites against pecan and peach fungal pathogens. In their study, 2 *P. luminescens* strains from 2 different *H. bacteriophora* strains, Hb and VS, also displayed variable effects with *P. luminescens* VS, causing a significantly higher growth inhibition to the pathogens, *Glomerella cingulata*, *Monilinia fructicola*, *Phomopsis* sp., *Phytophthora cactorum,* and *Fusicladosporium effusum.* Since the cell-free filtrate is a repertoire of many other metabolites, this heterogeneity might have also affected the specific anti-fungal metabolites' efficacy. Antimicrobial compounds in pure form are sometimes preferred over crude extracts. Some of the noteworthy metabolites of *Photorhabdus* that have been studied and proven to have antifungal activity in pure form in vitro include stilbene derivatives (Shi et al. 2017), trans-cinnamic acid (Bock et al. 2013), and benzaldehyde (Ullah et al. 2015).

## Conclusions

The in vitro inhibitory activity of local *P. luminescens* strains against *L. theobromae, F. oxysporum* f. sp. *lycopersici,* and *C.* species is only a preliminary proof of their biological control potential. Further bioefficacy assays coupled with the proper formulation and more in-depth molecular and biochemical studies could lead to the development of sustainable *Photorhabdus*-based biotechnology with a great utility to any integrated disease management programs in the Philippines.

#### Abbreviations

CFC: Cell-free culture filtrates; EPB: Entomopathogenic bacteria; EPN: Entomopathogenic nematode; NBTA: Nutrient bromothymol blue and triphenyltetrazolium chloride agar; PDA: Potato dextrose agar; TSB: Tryptic soy broth.

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#### Authors' contributions

RAL conceptualized the research idea and verified the experiments and analysis. SIRA and PMBR conducted the experiments and prepared the manuscript draft. BLC, along with RAL and SIRA, contributed to the final version of the manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

All data are available in the manuscript.

## Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no conflict of interest.

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