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Molecular identification and pathogenicity of *Phytophthora nicotianae*-caused bud rot disease of Washingtonia palms in Saudi Arabia and use of *Lysobacter enzymogenes* as a bioagent in an in vitro study

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Abstract

Severe terminal bud rot disease symptoms of Washingtonia, *Washingtonia robusta*, palms were observed in the 5-year-old trees in a farm located at the north of Al Shehia governorate, Qassim region, Saudi Arabia. The fungus found associated with the diseased palm buds was isolated in the laboratory, and pathogenicity tests were conducted on healthy 1-year-old Washingtonia palms, at an experimental station, Agriculture and Veterinary Medicine. Pathogenicity tests showed that a single fungus caused typical symptoms of bud rot on the inoculated trees. Petiole bases and terminal buds were rotted after 5–6 weeks of inoculation. The pathogen was identified through microscopy and characterized molecularly, using internal transcribed spacer (ITS) regions of ribosomal DNA, as a fungal-like organism *Phytophthora nicotianae* (synonym = *P. parasitica*). Experiments were conducted on the biological control of the pathogen in the laboratory, and a promising RN13 isolate of *Lysobacter enzymogenes* was selected for further study. This study seems to be the first report of bud rot disease of Washingtonian palms caused by *P. nicotianae* in Saudi Arabia. Great attention should be given to this disease because it is soil borne and may be transmitted to the date palm crop.

Keywords: Washingtonia palm, Bud rot, *Phytophthora nicotianae*, ITS region, *Lysobacter enzymogenes*, Biological control, Saudi Arabia

Background

Mexican Washingtonian trees, *Washingtonia robusta*, are a member of the family Arecaceae, which is native to Northwestern Mexico. It is one of the most regularly planted palm trees around the world as an ornamental plant (Broschat 2018). Although considered a desert palm, it is native to regions having permanent surface or subsurface water (Broschat 2018). A disorder resulting in the death of 4–5-year-old *W. roubsta* palms in a farm north of Al Shehia district, Al Qassim region, Saudi Arabia, was brought to our attention in March 2015. Many fungal species have been reported to cause bud roots in palm trees, including *Botryodiplodia*, *Chalara* (*Thielaviopsis*), *Colletotrichum*, and *Fuasrium*. *Phytophthora* species, oomycetes, also caused various diseases on various palms including seedling blights and damping off, trunk and leaf spots, crown and root rots, blights and petiole rots; nut drop; and apical, bud, or heart rot, followed by plant death (Garofalo and McMillan 1999). About 59 identified species of *Phytophthora* are known as pathogens causing disease in roots, crowns, stems, leaves, and fruits of a wide range of agricultural plant species (Erwin and Ribeiro 1996). *Phytophthora* is a

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soil-borne fungus-like organism from the water mold group (Ho 2018). *Phytophthora* bud rot is one of the most common diseases detected in palms in wet tropical climates (Garofalo and McMillan 1999). *Phytophthora nicotianae* (synonym = *P. parasitica*) has been reported to infect ornamental palms, more than 301 different ornamental plants and vegetable hosts such as onion, *Allium cepa*; carnation, *Dianthus caryophillus*; tomato, *Lycopersicum esculentum*; and poinsettia, uphorbia pulcherrima. It was recently reported to infect dwarf fan palm, *Chamaero pshumilis*, in Iran and Italy (Faedda et al. 2011 and Nazerian and Mirabolfathi 2013). Several *Lysobacter* spp. including *L. enzymogenes*, *L. antibioticus*, and *L. capsici* (Hayward et al. 2010) reported to have the potential as one of the biological control agents for plant diseases. 3.1T8 and OH11 strains of *L. enzymogenes* were very effective in inhibiting the mycelial growth of *Phytophthora capsici*, *Pythium ultimum*, *Py. aphanidermatum*, *Rhizoctonia solani*, *Fusarium graminearum*, *Ralstonia solanacearum*, and *Sclerotinia sclerotiorum* (Christensen & Cook, 1978, Folman et al. 2003, Jiang et al. 2005, and Qian et al. 2009).

The objective of this research was to survey the bud rot disease of Washingtonia palms caused by *P. nicotianae* and the isolation and identification by molecular methods based on sequencing of internal transcribed spacer (ITS) regions and to suggest a method for disease control.

Materials and methods

Sample collection and pathogen isolation

Three samples from symptomatic Mexican Washingtonian trees, *W. robusta*, displaying a dark brown rot on the petiole base and blight of the unopened spear leaves, were collected from Al-Salamah Farm in Alshehia district, Qassim region, Saudi Arabia. Symptomatic tissue was cut into small pieces (3 × 5 mm) and surface sterilized in 10% Clorox (NaOCl 0.5%) for 30 s, rinsed twice in a sterile distilled water, and blot dried on sterilized filter papers. Sterilized tissue was placed onto potato dextrose agar (CM0139, Oxoid, 39 g/L). Three pure cultures of fungi were obtained using mycelia tip culture after incubation at 25 °C for 5 days. Representative colonies of *Phytophthora* spp. were sub-cultured on V8 media (10%) according to Jeffers (2006), and the pure cultures were stored on corn meal agar in 8-ml glass vials at 15 °C in the dark.

Pathogenicity of *Phytophthora* spp. isolates

Pathogenicity tests were performed by wound inoculation with a cork borer of ten 1-year-old potted Washingtonian trees obtained from Qassim Baladiah nursery. After disinfection of the inoculation surface with ethyl alcohol (70%), a mycelial plug (5 mm diameter) of 7-day-old colonies of the pathogen grown on V8 media (10%) was inserted into the basal stem. Then, the hole was covered with the detached tissue and wrapped with Parafilm. All plants were incubated inside the humidity chamber at 24 ± 1 °C for 48 h with 100% relative humidity (RH) then transferred into the greenhouse at 25 ± 2 °C and 43% RH for 6 weeks (Faedda et al. 2011). A set of ten trees were inoculated for each isolate as well as for the untreated control. Plants were irrigated every 2 days with a tap water. The experiment was performed at the Agriculture and Veterinary Medicine Faculty Station in 2016.

Identification of the causal pathogen

Microscopic examination

Microscopic slide cultures were prepared periodically from the isolated fungi, and their hyphal and reproductive structures were examined, measured, and photographed by an Olympus light microscope (BX51TF, Japan).

Molecular identification

DNA extraction

The mycelium of three samples of *P. nicotianae*, Qassim isolates (Phyto1, Phyto2, and Phyto3), was harvested from the surface of 7-day-old potato dextrose agar plate cultures. One hundred milligrams of mycelial mate was grounded in liquid nitrogen and used for the extraction of total genomic DNA, using i-genomic plant DNA Extraction Mini Kit (iNtRON Biotechnology, Inc., Cat. No. 17371), following the manufacturer's instructions. The DNA was stored at − 20 °C.

Condition and amplification of PCR

Internal transcribed spacer (ITS) region amplification of rDNA was conducted in an automated thermal cycler (Swift TMMax Pro Thermal Cycler, ESCO healthcare), using ITS4/ITS5 primers (White et al. 1990). The PCR program used was 35 cycles of 94 °C for 30 s, 51 °C for 1 min, 72 °C for 1.5 min, and a final extension at 72 °C for 3 min. PCR mixture of 25 μl was as follows: 1 μl of 40 ng nucleic acid, 12.5 μl of GoTag®Colorless Master Mix (Promega Corporation, USA), 1 μl of each primer (10 pmol), and 9.5 μl of nuclease-free water (Promega). The amplified PCR products were electrophoresed for analysis through a 1.5% agarose gel containing 3 μl of ethidium bromide (10 mg/ml, Bio-Rad), and DNA bands were visualized using a UV transilluminator (G:BOXF3 system, Syngene).

DNA sequencing

The PCR products were purified, using QIA quick Gel Extraction Kit (Qiagen Inc., Chatsworth, California), and sent to Macrogen Inc. (Korea) for sequencing. The bands were sequenced in two directions with ITS4/ITS5 primers (White et al. 1990). Raw sequence chromatograms were gathered and modified, using GAP4

(Bonfield et al. 1995). Homologies to known sequences were identified, using the BLASTN algorithms (Altschul et al. 1997) compared to the redundant GenBank database at https://blast.ncbi.nlm.nih.gov/Blast.cgi. ClustalW was used for multiple alignments of sequences (Thompson et al. 1994), and the maximum parsimony method, using MEGA4 software, was used to draw the phylogenetic analyses (Tamura et al. 2007). The ITS4/ITS5 sequences of different *Phytophthora* species from several countries, used for comparisons, were retrieved from GenBank (www.ncbi.nlm.nih.gov).

Antifungal activity

Bacterial isolate

L. enzymogenes was selected among 20 other bacterial isolates from the previous study of Abdullah et al. (2016). This isolate was known as the best biocontrol agent against many plants pathogenic fungi compared to other tested isolates and coded RN13. This bacterium was identified by 16S rDNA as *L. enzymogenes* BB14 with 99% similarity percent, using BLAST (Abdullah et al. 2016).

In vitro antifungal activity

An overnight-grown bacterial culture was streaked as a circle shape on V8 media (10%) plate). A disk of *P. nicotianae* (phyto1 isolate) was placed in the middle of a bacterial circle and incubated at 25 °C for 7 days. V8 media (10%) plate was inoculated by fungus only as a control. The inhibition percent was recorded by measuring a growth diameter in treatment and control after the incubation period by following the formula according to Naureen et al. (2009): inhibition percentage = (diameter in control − diameter in treatment/diameter in control) × 100.

Results and discussion

Symptoms of the disease in the field

The diseased palms were individually scattered in the field, and the visual percentage of infected or dead palms was 3–5%. Symptomatology of the disease in the field included wilting of the youngest leaves, first manifested as a change in the leaf color to a paler green that progressed to a green brown and eventually to light brown as the leaves became desiccated and wilted (Figs. 1 and 2), and when making a cross section at the base of the diseased palm, the bases of the petioles show brown to dark brown soft tissues with a foul smell (Fig. 2). Progressive separation of the petiole bases of diseased palms showed tan-colored necrotic lesions, with brown margins. The surface of some lesions was covered by layers of white mycelia (Fig. 2). At the late stages of the disease, pale leaves completely died, buds were totally rotted, and leaf petioles disintegrated leaving a black cavity in the base of the dead palm (Fig. 3). Making a transverse section of a diseased tree at the petiole showed a brown discoloration and rot at the base and terminal bud tissues (Fig. 3). Bud (heart) rot in palm trees are caused by many species of *Phytophthora* in the world. *P. nicotianae* has been reported to infect blue Mediterranean fan palm (*Chamaerops humilis* var. *argenta*), causing basal leaf rot in Iran (Nazerian and Mirabolfathi 2013), in North America (Bomberger et al. 2016), and in Italy (Faedda et al. 2011). Therefore, bamboo palm, *Chamaedorea erumpens*; reed palm, *C. seifrizii*; Macarthur palm, *Ptychosperma macarthurii*; red sealing wax palm, *Cyrtostachys renda*; thatch palm, *Thrinax* sp.; and Mexican fan palm, *W. robusta* have been reported infected by *P. nicotianae* with stem, leaf, and root rots (Elliott et al. 2004). *P. palmivora* attacked *W. robusta* and *W. filifera*, causing one bud rot.

Pathogenicity test

All the three *Phytophthora* isolates (Phyto1, Phyto2, and Phyto3) were highly pathogenic to *W. robusta* young trees. Infected plants were pale, weathered, and growing poorly compared to inoculated plants 3 weeks after inoculation. The inoculated plants were almost dead at the fifth week compared to inoculated check plants (Fig. 4). When infected trees were longitudinally split by a sharp

Fig. 1 Symptoms of terminal bud rot of *Washingtonia robusta* trees in the field: diseased palms are individually scattered in the field (left) and wilted young leaves in the center and older pale green to brown (right)

Fig. 2 The transverse section in the base of diseased *Washingtonia robusta* tree (left) showing discoloration and rot of the petiole bases and terminal bud (middle) and brown lesions covered partially with white mycelium (right)

knife after 5–6 weeks from inoculation, the terminal bud and petiole bases were totally decayed showing brown to black rotted tissues (Fig. 5). Isolation from the dead and diseased tissues of the artificially inoculated trees revealed that all the tissues were infected with the same pathogen that was used for their inoculation.

Microscopic examination

The three isolated fungi were identical in their morphological characteristics. The pathogen was identified as *P. nicotianae* that was characterized by thick and branched hyphal growth (5–7.1 μm). Zoosporangia (30.36–43.97 μm) were formed on the mycelium 3–4 days after an incubation at 28 °C (Fig. 6a). Thickly walled sporangiospores (59.18–68.42 μm) were formed extensively on the mycelium in 7–15-day-old cultures (Fig. 6b). Sporangia were formed 3–4 days after an incubation at 28 °C and an extensive formation of chlamidospores was observed in culture plates of 7–15 days old. The results presented above are characteristics of *P. nicotianae* based on previous studies reported by Stamps et al. (1990) and Faedda et al. (2011). Traditionally, *Phytophthora* species taxonomic identification has been based upon microscopic examination of morphological characters and cultural criteria (Stamps et al. 1990).

P. nicotianae causes seedling blight of golden palm and also attacks California fan palm, causing trunk and collar rots. The disease appears to begin from wounds caused by leaf removal at or near the soil line. Although this disease can be reproduced by inoculations of the lower trunk, the bud and the entire stem are eventually rotted on some plants. Thus, *P. nicotianae* should also be considered as a possible cause of bud rots.

Molecular characterization of *P. nicotianae* isolates

A band of about 900 bp was amplified using total DNA from the three isolates (phyto1, phyto2, and phyto3) of *P. nicotianae* using the universal ITS4/ITS5 primers (Fig. 7). The three PCR products were sequenced obtaining for phyto1, phyto2, and phyto3 881, 843, and 843 bp, respectively. ITS region was conventionally used for the molecular identification of plant pathogenic fungi (Durán et al. 2010). The fungal ITS region varies between approximately 450 and 750 bp in length and consists of the variable spacers ITS1 and ITS2 and the intercalary 5.8S gene (Schoch et al. 2012). The ITS region sequences of *P. nicotianae* isolates were compared

Fig. 3 The late stage of the terminal bud rot disease of *Washingtonia robusta* tree showing complete decaying of the terminal bud (left) and total disintegration of tissues in the base of the tree leaving a black hallow cavity in the base of the dead tree

Fig. 4 Terminal bud rot symptoms of a *Washingtonia robusta* tree inoculated with *Phytophthora nicotianae* 5 weeks after inoculation (left and middle) compared to non-inoculated tree (right)

by other ITS sequences of *Phytophthora* species reported in GenBank (www.ncbi.nlm.nih.gov). The phylogenetic results (Fig. 8) revealed that the *Phytophthora* isolates formed two clades (I and II). Clade I includes the three *P. nicotianae* isolates from Qassim region and all *P. nicotianae* and *P. parasitica* isolates. However, the three *P. nicotianae* isolates from Qassim region have been separated and formed subclade B with *P. nicotianae* isolate 2013CAP2002 (KJ549640) from the USA and *P. nicotianae* strain IIFT 155 (GU073389) from Cuba with 100% of the identity confirming genetic similarity of isolates (Fig. 8). *P. palmivora* strains were distanced from other *Phytophthora* isolates by bootstrap 100% and grouped together in clade II. The Qassim *P. nicotianae* isolates had more than (99.6%) nucleotide identity with *P. nicotianae* and *P. parasitica* isolates, whereas the identities were less than (89.3%) with all *P. palmivora* strains. An analysis of nucleotide sequences of internal transcribed spacer (ITS) regions of rDNA has been used to differentiate *Phytophthora* species (Cooke et al. 2000, Förster et al. 2000, and Meenupriya and Thangaraj 2011). Also, the ITS could be successfully used for discriminating closely related species of a broad range of fungi (Schoch et al. 2012).

Fig. 5 Splitting of a diseased plant (left) showing internal symptoms of terminal bud rot on 1-year-old *Washingtonia robusta* trees artificially inoculated with *Phytophthora nicotianae* and maintained in a greenhouse for 5 weeks. A non-inoculated tree (right)

Antifungal activities

As shown in Fig. 9, the growth of *P. nicotianae* (Phyto1 isolate) was more inhibited by using the bacterial strain *L. enzymogenes* BB14 than in the control treatment. The tested bacterial isolate exhibited stronger inhibitory effects on mycelial growth of *P. nicotianae* after 7 days, nearly 100% inhibition percentage. The potential biological control for plant diseases has been recently reported, using *Lysobacter* species (Folman et al. 2003 and Sullivan et al. 2003). Li et al. (2008) reported that *L. enzymogenes* strain C3 is a biological control agent that has a strong antagonist effect against several fungal pathogens. *L. enzymogenes* has been reported to suppress soil-borne diseases, such as *Rhizoctonia solani* that caused brown patch in turf grass (Giesler and Yuen 1998). Moreover, *L. enzymogenes* has been cited to control foliar diseases, for example, *Bipolaris sorokiniana* causal agent of leaf spot of tall fescue (Zhang and Yuen 1999), *Uromyces appendiculatus* causal agent of bean

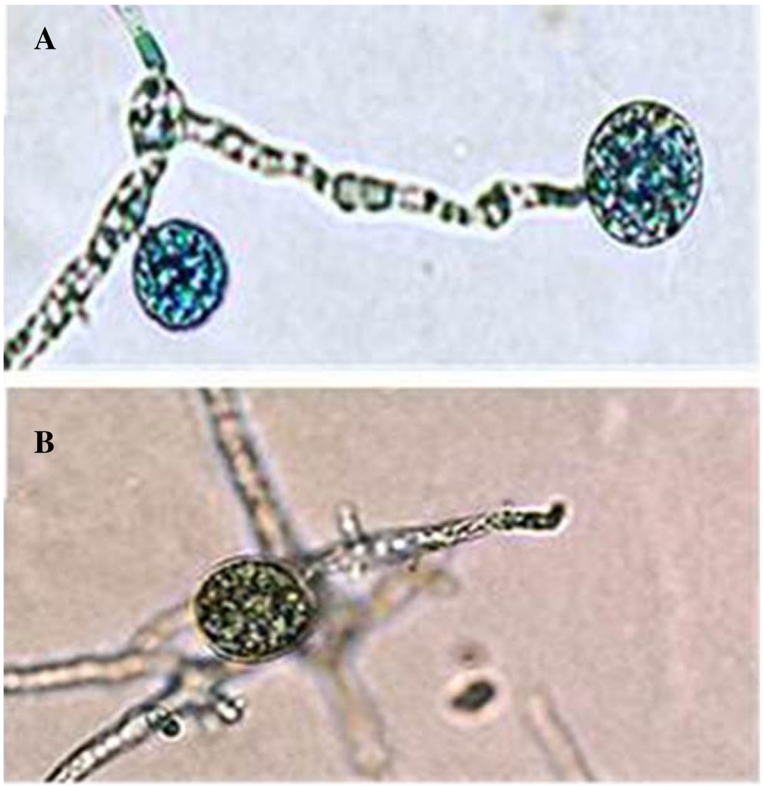

Fig. 6 a Sporangia (30.36–43.97 μm) and **b** sporangiospores (59.18–68.42 μm) of *Phytophthora nicotianae*

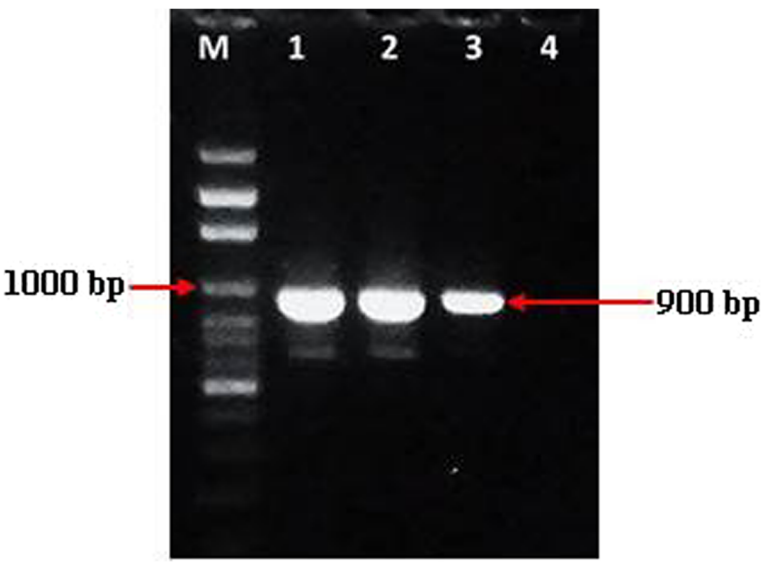

Fig. 7 Agarose gel electrophoresis of polymerase chain reaction of ITS region using ITS4/ITS5 primers. Lanes 1, 2, and 3 are Phyto1, Phyto2, and Phyto3 isolates of *Phytophthora nicotianae*, respectively. Lane 4 is water, and M is a 100-bp DNA ladder (Solis BioDyne)

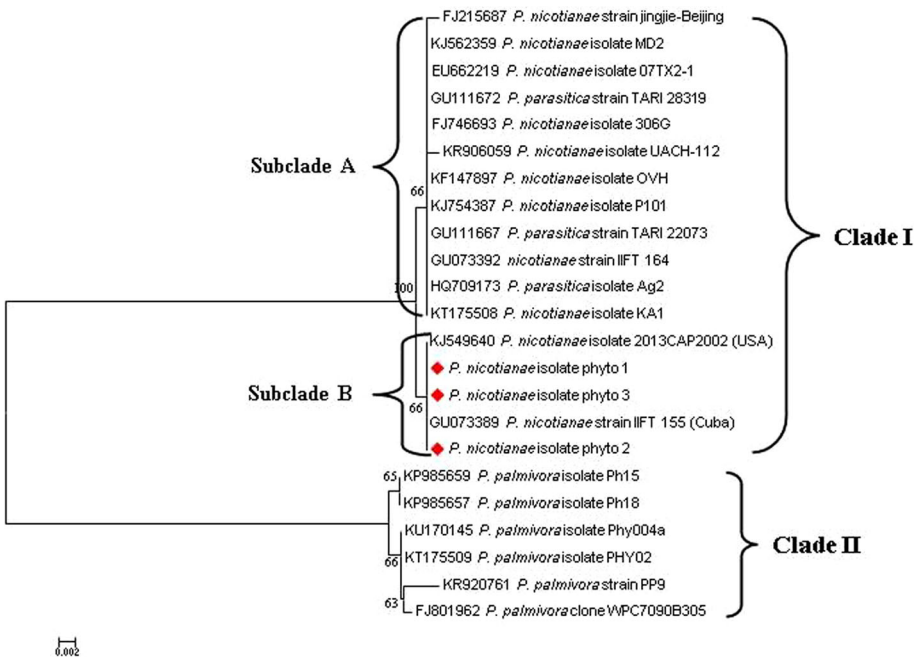

Fig. 8 Phylogenetic tree derived from the internal transcribed spacer (ITS) region sequences of *Phytophthora nicotianae* and other *Phytophthora* spp. isolates using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. GenBank accession numbers for sequences are written in the left beside the *Phytophthora* sp. Phylogenetic analyses were conducted in MEGA4

rust (Yuen et al. 2001), and *Fusarium* head blight of wheat (Jochum et al. 2006).

In this study, the bacterial isolate *L. enzymogenes* BB14 was more active in chitin hydrolysis as shown in Fig. 10 which gives it more antifungal activity against *P. nicotianae*. Li et al. (2008) mentioned that the antifungal activity of *L. enzymogenes* was attributed in part to lytic enzymes. Many extracellular enzymes that contribute to biocontrol activity, including multiple forms of β-1, 3-glucanases, and chitinases, are produced by *L. enzymogenes* as reported by Zhang et al. (2001) and Palumbo et al. (2005).

Conclusion

Terminal bud rot of *W. robusta* observed in Al Qassim region, caused by *P. nicotianae*, is a pathogenic disease that could be transmitted to other crops through infected soil and irrigation water as well as farming tools. The pathogen is similar in morphology and disease

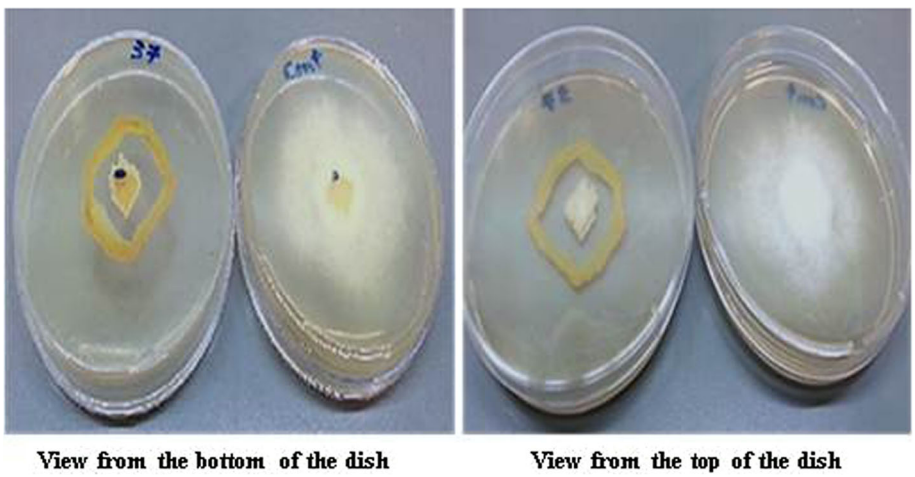

Fig. 9 The growth of treated *Phytophthora nicotianae* by *Lysobacter enzymogenes* BB14 compared to that of untreated fungus on V8 media (10%) plates

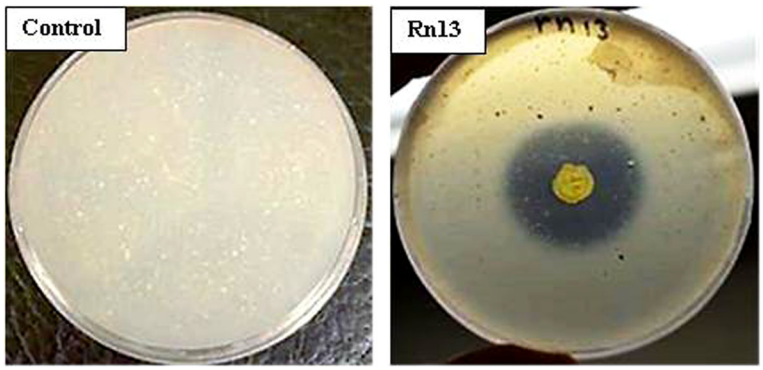

Fig. 10 Chitin hydrolysis by the bacterial isolate RN13 (*Lysobacter enzymogenes* BB14) on CCA medium

symptoms to *P. palmivora*, but the sequencing analyses of internal ITS regions of ribosomal DNA confirmed that the causal agent was *P. nicotianae*. Experiments on the biological control of the disease showed that the bacterial strain *L. enzymogenes* was a potential antagonist to *P. nicotianae* on V8 media (10%) Petri plates. However, further studies are needed on the control of the disease and susceptible plant species both in the laboratory and in the field.

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Authors' contributions
All of the authors of this manuscript contributed equally to the design and/or execution of the experiments described in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
We give our consent for the publication in Egyptian Journal of Biological pest Control.

Competing interests
The authors declare that they have no competing interests.

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