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The potential of endophytic fungi as bio-control agents against the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

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Abstract

Endophytes are promising bio-control agents and rich sources of secondary metabolites known for their biological activities. Two medicinal plants, *Pelargonium graveolens* and *Melia azedarach*, and two weeds, *Chenopodium album* and *Malva parviflora*, were selected to isolate endophytic fungi. Spore suspension of *Alternaria alternata* (MG786545), *Alternaria solani* (MG786543), *Curvularia lunata* (MF113056), *Nigrospora sphaerica* (MF113055), and *Stemphylium* sp. endophytes was tested for their pathogenicity to the third-instar larvae of the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Two techniques were used: dipping in spore suspension and residual film assay. Secondary metabolites were extracted, and ethyl acetate extracts of *A. alternata* (MG786545), *C. lunata* (MF113056), and *N. sphaerica* (MF113055) were tested for their insecticidal activity by both techniques. *C. lunata* (MF113056) showed the most potent pathogenicity causing 60% mortality after 7 days by dipping bioassay, while *A. solani* (MG7865453) and *A. alternata* (MG786545) caused 40 and 33.3% mortality, respectively. All tested endophytes showed no effect against *S. littoralis* by residual film method. *N. sphaerica* ethyl acetate extract activated larval feeding on castor leaves causing 25% mortality by residual film at 2000 mg/l.

Keywords: Endophytes, Fungal metabolites, *Spodoptera littoralis*, Residual film, Biological control

Background

The extensive use of conventional pesticides has led to serious ecological problems including hazards to human health and animals, resistance of target pests, and environmental pollution. Bio-pesticides are natural pesticides derived from animals, plants, microorganisms, and certain minerals. They are eco-friendly tools of managing agriculture pests, less toxic, and specific on target organisms (Gupta and Dikshit 2010). Microbial pesticides' active ingredients are microorganisms such as fungus, bacterium, virus, protozoan, or algae that have a broad spectrum of activity, but every organism is relatively specific to its target pest (Lord 2001). *Beauveria bassiana* is one of the most common fungal examples of successful entomopathogenic used as a bio-control of several insect pests and has been registered by the US Environmental Protection Agency (EPA) as a bio-control agent for a wide range of insect pests (Salma et al. 2011).

Endophytes are microorganisms that grow within plants without causing any obvious symptoms of infection or disease. Some endophytes accelerate the growth of their host plants and increase nutrient uptake (Hashem et al. 2016). Other endophytes protect their hosts by producing secondary metabolites which have the potential to kill or suppress the attack by pathogenic microorganisms or herbivores (Carroll 1988 and Schulz et al. 2002). So, endophytes can be used as one of the biological control agents in sustainable crop production (Rybakova et al. 2016 and Egamberdieva et al. 2017).

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), is a serious lepidopteron pest in subtropical and tropical regions. It has a wide range of host plants belonging to 44 different

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families including grasses, legumes, and cruciferous and deciduous fruit trees (Abdel-Megeed and Iss-Hak 1975).

The aims of this work were to isolate endophytic fungi from Egyptian weeds and medicinal plants, to extract their secondary metabolites, and to evaluate their potential against *S. littoralis* larvae.

Materials and methods

Isolation of endophytic fungi

Root samples of *Malva parviflora* and leaf samples of *Chenopodium album* and *Pelargonium graveolens* were collected from Abees farm, and leaf samples of *Melia azedarach* were collected from the garden of the Faculty of Agriculture, Alexandria, Egypt. The samples were washed with tap and distilled water, air dried, surface sterilized by 70% ethanol for 1 min then by 3.0% sodium hypochlorite (NaOCl) for 3 min, and rinsed with sterile distilled water for 1 min three times. The surface-sterilized samples were cut into 5 mm^2 surface using a sterile blade and placed in Petri dishes with potato dextrose agar media containing 50 mg/l of ampicillin (Kharwar et al. 2012). Each plate contained four segments of plant pieces with four replicates for each plant. The dishes were incubated at 25 ± 2 °C and observed daily to record the emergence of endophytes and pre-cultured on potato dextrose agar (PDA) media. For testing the effectiveness of surface-sterilized plant samples, 1 ml of the last rinsing water was placed on the PDA media and incubated at 25 ± 2 °C.

Identification of fungal isolates

Morphological observations were carried out, using both macroscopic and microscopic characters, for endophytic fungus culture and spore shapes and types, using the standard manual of Barnett and Hunter (1998). The isolated endophytes were subjected to DNA extraction, using the Qiagen DNA extraction kit (Qiagen, Germany). According to Hafez and Elbestawy (2009), endophytic fungi were identified by amplification of ITS1-5.8SrRNA-ITS4 gene using the universal primers: ITS1 (forward primer: 5-TCC GTA GGT GAA CCT GCG G-3) and ITS4 (reverse primer: 5-TCC TCC GCT TAT TGA TAT G-3). PCR reaction mixture (25 μl) consisted of 12.5 μl PCR Master Mix (2X) Promega Corporation, 1 μl (50 pmol) of each oligonucleotide primer, 1 μl DNA template, and 9.5 μl nuclease-free water. The reaction cycles consisted of initial denaturation (95 °C, 2 min), 35 cycles of denaturation (95 °C, 31 min), annealing (55 °C, 1 min), extension (72 °C, 1 min), and final extension (72 °C, 10 min). The PCR products were analyzed using agarose gel electrophoresis with ethidium bromide (EtBr) staining. The products were purified using the purification kit (Qiagen) and sequenced by DNA sequencer by LGC group (Berlin, Germany). After preprocessing, the

resulting DNA sequence was identified using the BLASTN tool of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and nucleotide collection (nr/nt) database. To create the phylogenetic tree of the endophytic fungi, advanced version of the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 was used (Tamura et al. 2013).

Pathogenicity of endophytic fungi spore suspension

By dipping technique

The endophytic fungi grown in PDA plates with high spore production levels (*Alternaria alternata* (MG786545), *A. solani* (MG786543), *Curvularia lunata* (MF113056), *Nigrospora sphaerica* (MF113055), and *Stemphylium* sp.) were flooded by sterile distilled water containing 0.05% Triton X-100, scrapped with surface-sterilized spatula, and filtered through cheesecloth to remove mycelial debris. Spore suspensions were prepared, and their concentrations were adjusted to 10^8 spores ml^{-1} by a hemocytometer then tested against third-instar larvae of *S. littorals*. Ten larvae were dipped in spore suspensions for 5 s and left to dry on a filter paper. Two control treatments were conducted: with sterilized distilled water and with aqueous solution of 0.05% Triton X-100. The treated larvae were transferred to glass bottles containing fresh castor leaves, with four replicates for each treatment (Baskar et al. 2012). The experiment was checked daily for 2 weeks to keep clean, the castor leaves were changed, the dead larvae were counted, and the mortality percent was calculated. The dead larvae were surface sterilized and placed on the Petri dishes containing PDA to confirm if the mortality was due to the fungi pathogenicity.

By residual film

The same concentration of spore suspensions used for the previous bioassay was used for residual film bioassay. Castor leaves were submerged in spore suspensions for 20 s, left to dry, and transferred to glass bottles with ten starved (4–5 h) third-instar larvae of *S. littoralis*. Castor leaves treated with sterilized distilled water and with sterilized distilled water containing 0.05% Triton X-100 served as the control treatments with four replicates for all treatments. The results were taken as described above.

Extraction of endophytes secondary metabolites

Based on the results of the previous pathogenicity bioassay, three endophytic isolates from different genera were chosen for fermentation and extraction of their secondary metabolites. Five disks of each endophytic fungus were inoculated in 1 l Erlenmeyer flask containing 300 ml potato dextrose broth (3 l PDB) and incubated at 28 °C for 2 weeks in a shaker incubator at 120 rpm. The culture broth was centrifuged

for 10 min at 15000×*g*, and the supernatant was extracted by the solvent mixture (ethyl acetate to methanol to water, 8:4:1) in an ultrasonic bath at 40 °C for 20 min. The solvent layers were separated by a separatory funnel, and the aqueous layer was extracted twice by ethyl acetate with sonication for 20 min. The ethyl acetate layer was collected and evaporated under vacuum to dry and kept at 4 °C for bioassay.

Pathogenicity of endophyte secondary metabolites
Ethyl acetate extracts were tested against third-instar larvae of *S. littoralis* by dipping and residual film techniques (Pampapathy et al. 2011). Concentrations of ethyl acetate crude extracts (250, 500, 1000, 2000 mg/l) were prepared in a sterile distilled water containing 0.05% Triton X-100. Ten larvae of *S. littoralis* were dipped in each extract concentrations for 5 s, left on filter paper for 10 s to dry, and transferred to the Petri dishes containing castor leaves, with four replicates.

The same concentrations of ethyl acetate extracts were used for residual film bioassay. Castor leaves were dipped in each extract concentrations for 20 s, left to dry, and transferred to the Petri dishes containing ten starved (4–5 h) larvae of *S. littoralis* with four replicates. Control treatments were conducted using castor leaves treated with sterilized distilled water and with sterilized distilled water containing 0.05% Triton X-100. Mortality rate was observed daily for 1 week, and mortality percent was calculated.

Statistical analysis
Percentage of *S. littoralis* mortality was subjected to one-way analysis of variance followed by Student-Newman-Keuls test (Cohort Software Inc. 1985) to determine significant differences between mean values at the probability level of 0.05.

Results and discussion
Identification of the endophytic isolates
Fungal identification by morphological characters resulted in the identification of ten endophytic fungi. Eight of them were identified by DNA sequences of the purified PCR product. Five endophytic fungi were isolated from *C. album* weed: *F. chlamydosporum* saad3 MG786540, *A. alternata* saad5 MG786542, *A. alternata* saad8 MG786545, *F. oxysporum* saad4 MG786541, and *Phoma* sp. Two were isolated from *M. parviflora* weed: *F. equiseti* saad7 MG786544 and *Stemphylium* sp. Two were isolated from the medicinal plant *M. azedarach*: *C. lunata* saad2 MF113056 and *N. sphaerica* saad1 MF113055. The last one was isolated from the aromatic plant *P. graveolens*: *A. solani* saad6 MG786543 (Table 1). Figures 1 and 2 illustrate the gel electrophoresis and phylogenetic tree of the isolated endophytic fungi, respectively.

Pathogenicity of endophytic fungi and their metabolites
The results of spore suspension bioassays indicated that the mortality of *S. littoralis* larvae differed according to the fungus species and application method. The endophytic isolate pathogenicity was confirmed after the fungi mycelium emerged from the dead larvae (Fig. 3). In dipping method, *C. lunata* (MF113056) spore suspension showed the most virulence with 60% mortality rate after 7 days. *A. solani* (MG7865453) and *A. alternata* (MG786545) caused 40% and 33.3% mortality rate, respectively, but there was no significant difference between them. In contrast, *N. sphaerica* showed no virulence by both techniques (Fig. 4). The mortality did not increase after 1 week until the end of the experiment for all used endophytes. All the tested endophytes showed very weak or no pathogenicity by residual film method, and there was no difference between endophyte treatments and control treatments regarding larval mortality. The ethyl acetate extracts showed no effects in *S.*

Table 1 Endophytic fungi isolated from plant leaves and roots and their accession numbers

| Plant	Plant part	Endophytic isolates	Accession number
Melia azedarach	Leaf	*Nigrospora sphaerica*	MF113055
		Curvularia lunata	MF113056
Chenopodium album	Leaf	*Fusarium chlamydosporum*	MG786540
		F. oxysporum	MG786541
		Alternaria alternata	MG786542
		A. alternata	MG786545
		Phoma sp.	–
Malva parviflora	Root	*F. equiseti*	MG786544
		Stemphylium sp.	–
Pelargonium graveolens	Leaf	*A. solani*	MG786543
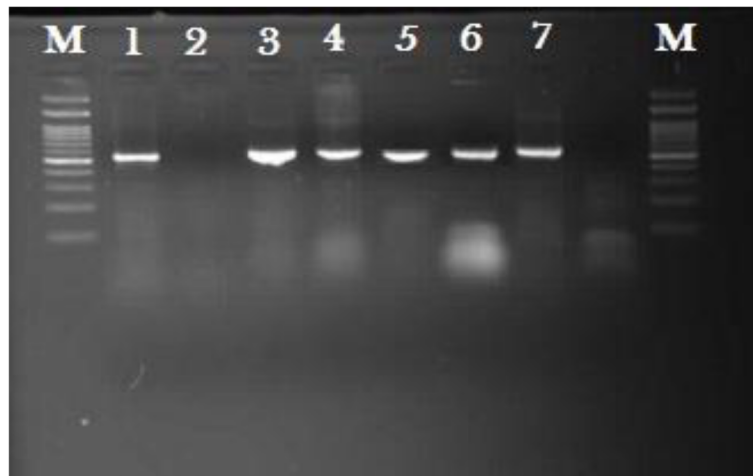
Fig. 1 Gel electrophoresis shows (from right to left) DNA ladder fragments of (1) *Fusarium equiseti*, (3) *F. chlamydosporum*, (4) *F. oxysporum*, (5) *Alternaria alternata*, (6) *A. alternata*, and (7) *A. solani*

littoralis mortality by dipping method and weak effects by residual film method. Only ethyl acetate crude extract of *N. sphaerica* activated the feeding on castor leaves and caused 25% mortality rate by the residual film method at 2000 mg/l (Fig. 5).

Many researchers have reported the endophytic fungi as pathogenic to insects and can protect their host plants from herbivores by producing secondary metabolites (Schardl 2001; Baskar et al. 2012; and Bensaci et al. 2015). Senthilkumar et al. (2014) found
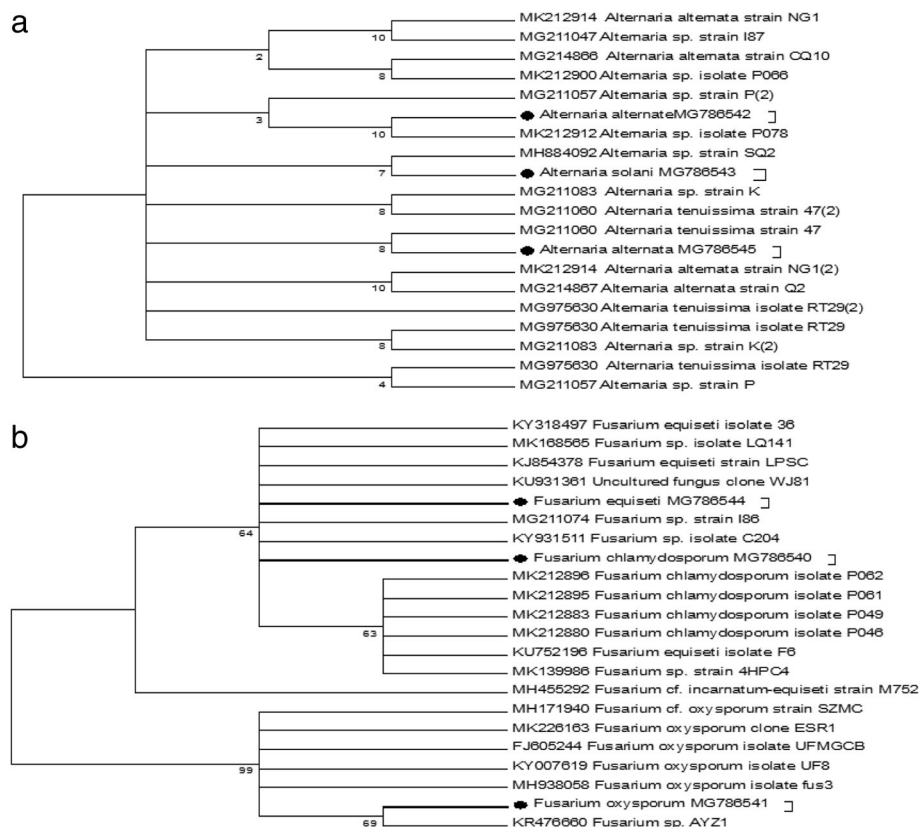
Fig. 2 Phylogenetic tree based on the alignment of nucleotide sequences of ITS region of rDNA gene among selected endophytic fungal isolates (**a**) *Alternaria* sp, (**b**) *Fusarium* sp isolated from the leaves of both *Chenopodium album* and *Pelargonium graveolens* and roots of *Malva parviflora*

Fig. 3 Fungal hyphae emerged from the cadaver of *Spodoptera littoralis* larvae treated with spore suspension of *Curvularia lunata* by dipping method

that the methanol extracts (1000 ppm) of *Aspergillus flavus* and *Nigrospora sphaerica* isolated from *Tectona grandis* caused 65% and 62% mortality of *Hyblea purea*, 59% and 56% of *Eligma narcissus*, and 46% and 42% of *Atteva fabriciella*, respectively. Han et al. (2013) stated that ethyl acetate extracts of both 16 strains mycelium and 9 strains fermentation broth showed a larvicidal activity against the third-instar larvae of *Culex pipiens pallens* mosquitos. These

active strains were focused in the genera *Aspergillus*, *Penicillium*, *Phoma*, and *Pesalotiopsis*. However, the obtained results showed that *C. lunata* (MF113056) and *A. alternata* (MG786545) had the potential to infect and cause the death of *S. littoralis* larvae by direct contact not by consuming fungal spores or by their metabolites. This finding is in agreement with the entomopathogenic fungal mechanism of action, as it invades directly through the cuticle, mechanically
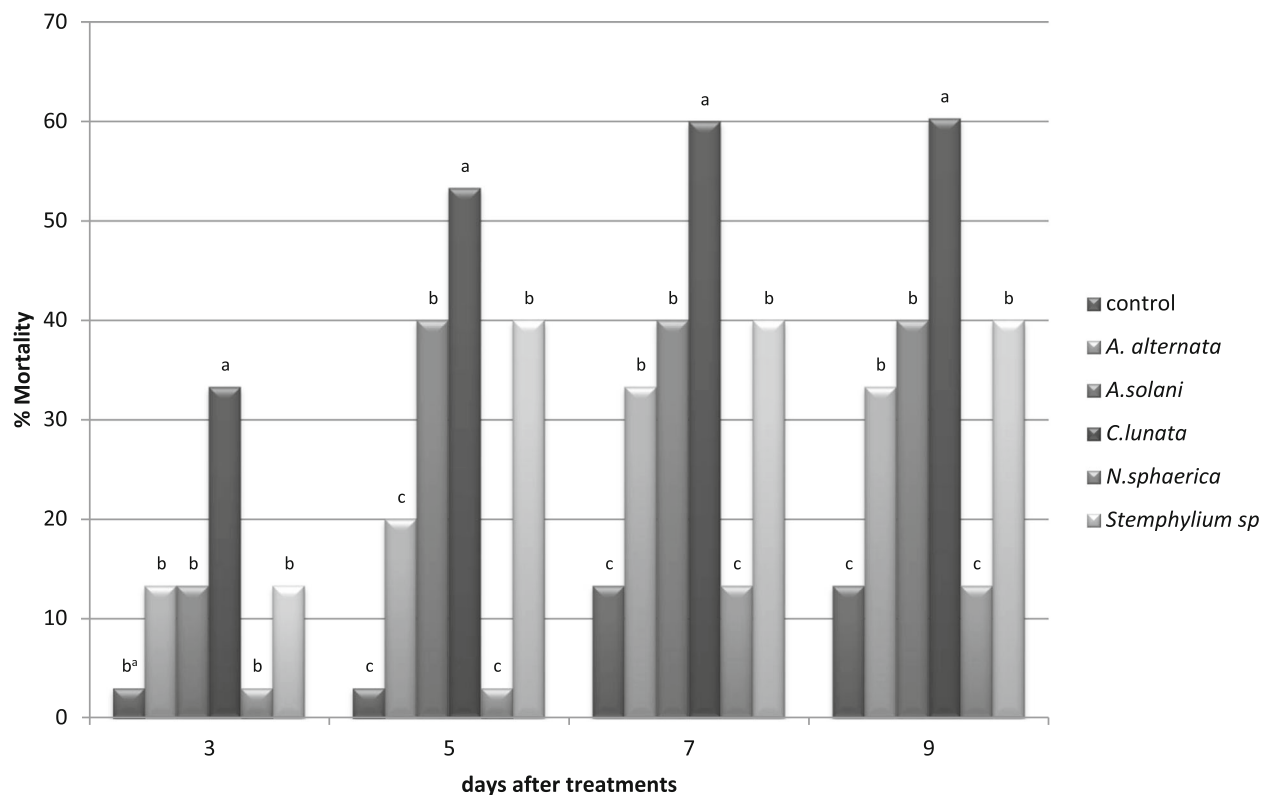

Fig. 4 Pathogenicity of endophytic fungi on third-instar larvae of *Spodoptera littoralis* by dipping in spore suspension (10^8 spores/ml). (a) Percent mortality within the same day sharing the same letter is not significantly different at the 0.05 probability level
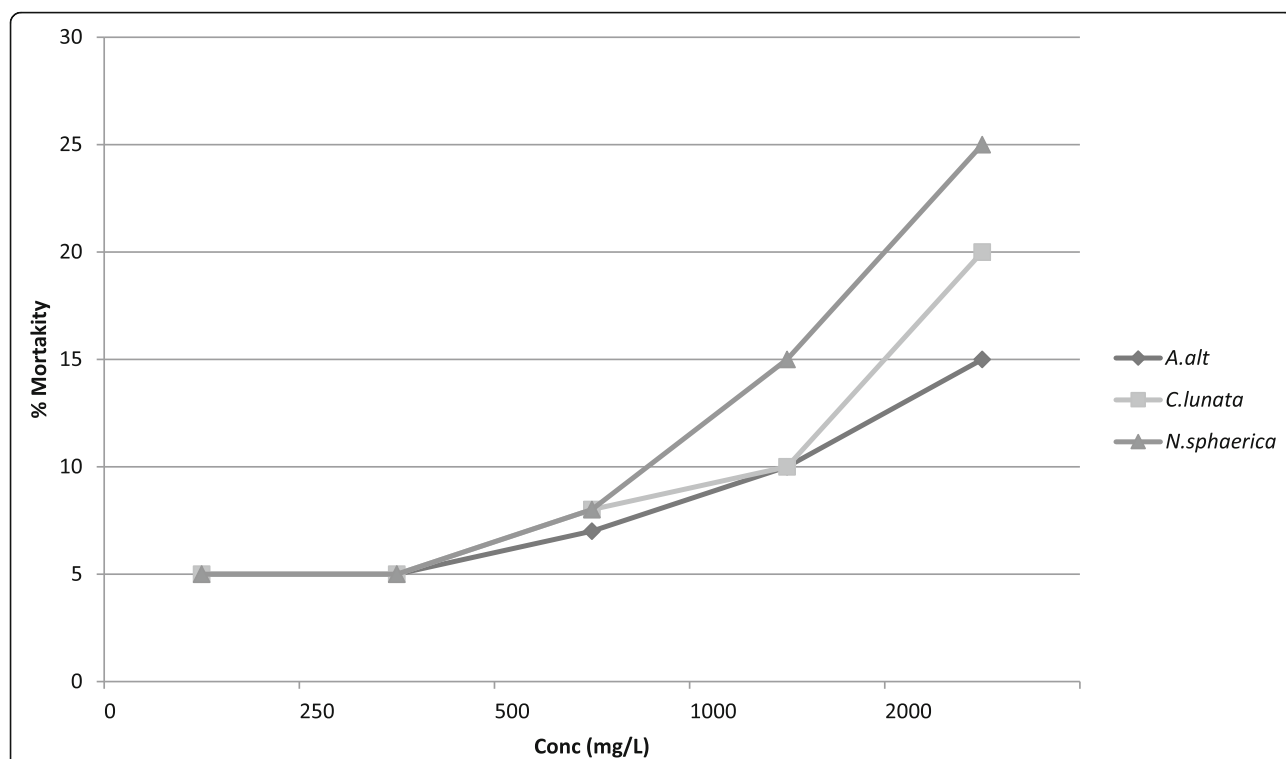
Fig. 5 Mortality percent of the third-instar larvae of *Spodoptera littoralis* caused by ethyl acetate extracts of the endophytic fungi by residual film after 5 days

or by enzymatic production without a need to be consumed by their host to cause infection (Ownley et al. 2008, 2010, and Vega 2008, Vega et al. 2012).

In conclusion, the obtained finding showed the potential of *C. lunata* (MF113056), *A. solani* (MG7865453), and *A. alternata* (MG786545) to act as entomopathogenic fungi and cause 60%, 40%, and 33.3% death of *S. littoralis* larvae, respectively. Therefore, endophytic fungi represent a group of organisms with a potential for applications as one of the bio-control agents against insect pests.

Availability of data and materials
Not applicable in this section.

Authors' contributions
All authors contributed to the design of the experiment, acquisition of data, analysis and interpretation of data, and drafting and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable in this section.

Consent for publication
Not applicable in this section.

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

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