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Establishment of *Beauveria bassiana* as a fungal endophyte in pecan (*Carya illinoinensis*) seedlings and its virulence against pecan insect pests

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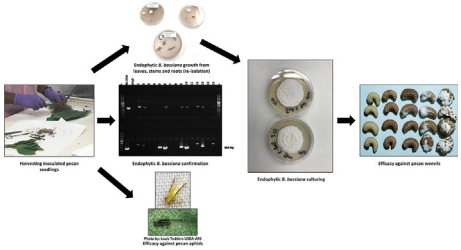
GRAPHICAL ABSTRACT



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ABSTRACT

Pecan (*Carya illinoinensis*) is an important crop in the USA, which produces approximately 55% of the world's pecans. Several insect pests and various plant diseases reduce crop yield directly destroying nutmeats, or indirectly through effects on foliage and shucks, reducing photosynthesis. *Beauveria bassiana* is a well-studied, commercialized fungal entomopathogen that when applied inundatively is an effective biocontrol agent against certain pecan pests. In addition to being used in inundative biocontrol, *B. bassiana* can exist as an endophyte in many plant species and has been shown in some cases to reduce pest damage when present as an endophyte. The potential for *B. bassiana* to exist as an endophyte in pecan had not been explored previously. We tested whether *B. bassiana* could endophytically colonize pecan seedlings by seed soaking, seed coating and soil drenching. Results indicated that *B. bassiana* became established in the roots, leaves and stems of pecan seedlings. Establishment was verified using molecular techniques as well as completing Koch's postulates on the re-isolated fungus, infecting two susceptible insect hosts (*Galleria mellonella* and *Tenebrio molitor*) and a target pest (*Curculio caryae*). Subsequently we explored whether the established endophytic fungus suppressed two pecan aphid species. In a leaf-disc assay, populations of two pecan aphids (*Melanocallis caryaefoliae* and *Monellia caryella*) were reduced when placed on leaves of pecan that were colonized endophytically with *B. bassiana*, relative to control leaves. Our study demonstrates the ability to establish endophytic *B. bassiana* in pecan and the potential to apply this capability in pecan pest management. Additional research is needed to determine the utility of the endophytic approach against various insect and plant pathogens and to measure efficacy under field conditions.

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1. Introduction

Pecan, *Carya illinoinensis* is an important economic crop in the United States of America (USA), with an estimated value of ca. $560 million and approximately 55% of the world's production (Kim and Dharmasena, 2018). Several insect pests and various plant diseases reduce crop production potential directly destroying nutmeats, or indirectly through effects on foliage and shucks, reducing photosynthesis (Smith et al., 2019). The most important insect pests of pecan are: the black pecan aphid, *Melanocallis caryaefoliae* (Davis) (Hemiptera: Aphididae) together with other aphid species significantly reduce kernel quality and greatly reduce the subsequent year's crop; pecan weevil, *Curculio caryae* (Horn) (Coleoptera: Curculionidae), which can damage up to 90% of nutmeats; and stink bugs (Hemiptera: Pentatomidae) (Dutcher 1991; Shapiro-Ilan et al., 2013; Hatting, 2018).

The main pest control measure growers have relied on is application of conventional pesticides at calendar intervals (Shapiro-Ilan et al., 2013; Smith et al., 2019). The use of pesticides is discouraged due to the development of resistance, chemical residues and the toxicity of pesticides to other, non-target organisms and the environment (Lacey and Georgis, 2012). The trend is for use of safer and more environmentally benign products. The use of biological control products comprises an important alternative to conventional pesticides. Foliar and/or soil applications of fungal entomopathogens (e.g., *Beauveria*, *Metarhizium*, *Isaria*, *Lecanicillium* and *Hirsutella*) have been used in an attempt to manage agricultural insect pests (Skinner et al., 2014). A well-characterized fungal entomopathogen is *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycota: Hypocreales) (de Faria and Wraight, 2007). Although *B. bassiana* is widely commercialized, its application is limited by adverse environmental conditions including UV light, low moisture, etc. (Wraight et al., 2007; Vega et al. 2012). Generally, inundative application of biocontrol agents rely on the direct action of the released agent without or with little secondary effect on successive pest generations (Vincent et al., 2007). As a result, much recent research is aimed at introducing fungal entomopathogens as endophytes. Vega (2018) reported that whereas five articles on endophytic fungal entomopathogens were published between 1990 and 1999, there were 20 articles published between 2000 and 2009, and 60 between 2010 and 2018, thus showing an increased interest on this topic.

Fungal endophytes are defined as "fungi inhabiting plant tissues without causing symptoms or harm in the host plant" (Wilson, 1995). They have been demonstrated to protect plants against herbivorous insects and plant pathogens (Jallow et al., 2004, 2008; Ownley et al., 2008). Jaber and Ownley (2018) and Vega (2018) reviewed studies of different fungal entomopathogens that can exist as endophytes and suppress insect pests, plant pathogens, and act as growth promoters with beneficial rhizosphere colonization in various plant species. *Beauveria bassiana* has been successfully inoculated and established as an endophyte in 25 different plant species, and has also been shown to have activity against insect pests and plant pathogens (Vega, 2018). These studies reported successful establishment of *B. bassiana* as an endophyte by either seed treatment, soil application, direct injection and/or roots drenching, with subsequent control of insect pests and/or beneficial effects on plant growth.

To date, no study has determined whether fungal entomopathogens can exist as endophytes in pecan trees, and suppress insect pests and plant pathogens. If successfully established, endophytic *B. bassiana* has potential to reduce the impact of pecan insect pests and plant pathogens, including species of aphids [black pecan aphid, blackmargined aphid, *Monellia caryella* (Fitch), and yellow pecan aphid, *Monelliopsis pecanis* (Bissell), pecan weevil and stink bugs.

The objectives of the current study were to determine: 1) whether *B. bassiana* can colonize pecan seedlings from artificial inoculation by nut soaking (wet or dry) and soil drench; 2) whether endophytic *B. bassiana* cultured out from artificially inoculated pecan seedlings retains the entomopathogenicity of the original inoculum; 3) whether the established endophytic fungus is effective for suppression of pecan aphids.

2. Material and methods

2.1. Source of fungal inoculum

Beauveria bassiana strain GHA (BotaniGard 22WP, 2×10^{13} viable spores per pound) was purchased from Emerald BioAgriculture (Salt Lake City, UT) and subcultured on Sabouraud dextrose agar with 0.2% yeast extract (SDAY; Fisher Scientific, Waltham, MA) according to procedures described by Goettel and Inglis (1997). The fungus was cultured for 14–18 days, when conidia were harvested by scraping the agar surface with a sterile spatula. Conidial concentrations were determined using an improved Bright-Line™ Hemacytometer (Hausser Scientific, Horsham, PA) and the suspensions were adjusted to 1×10^8 conidia ml^{-1} in sterile distilled water containing 0.05% Silwet L-77 (Fisher Scientific, Waltham, MA) according to Parsa et al. (2013). For all experiments, conidial viability was evaluated by taking a 100 ml sample of each inoculum, plating it on potato dextrose agar (PDA; Difco, Sparks, MD), incubating at 25 °C for 24 h in the dark and assessing germination under an Olympus BX51 light microscope (Olympus Corporation, Center Valley, PA) by counting germinated spores from a total of 100 randomly selected conidia. Conidia were deemed to have germinated if the germ tube was at least twice the length of the conidia. Only inocula with germination of ≥90% were used for the experiment.

2.2. Fungal inoculation of pecan nuts and seedlings

2.2.1. Nut sterilization prior to fungal inoculation

Nuts were surface sterilized according to Parsa et al. (2016). Briefly, sixty to 100 nuts were surface sterilized by immersing in 0.05% Silwet L-77, followed by immersion in 0.5% sodium hypochlorite, and finally 70% ethanol, for 2 min each. The seeds were rinsed three times in sterile distilled water in a laminar flow hood and allowed to dry on sterile paper towels. The effectiveness of the seed surface sterilization method was evaluated by plating 100 µl aliquots of the rinsate and pressing and rolling individual nuts on PDA in 100 × 15 mm Petri dishes. Plates were incubated at 25 °C for 10 d in darkness. The disinfection was considered successful if no microbial growth was observed on the surface of the PDA. Nuts were discarded if any microbial growth was observed.

2.2.2. Nut inoculation by soaking

Surface sterilized nuts were soaked for 24 h in 300 ml of a suspension of *B. bassiana* strain GHA (1×10^8 conidia ml^{-1} containing 0.05% Silwet L-77), based on the method described by Castillo Lopez and Sword (2015). Control nuts were soaked in sterile water. Beakers containing the soaking nuts were placed in an incubator in the dark at 25 °C until planting the following day. Nuts were planted in 15.2 × 15.2 cm plastic pots using sterilized soil (loamy sand; 84% sand, 10% silt, 6% clay; 2.8% organic matter; pH = 6.1) and were placed in a greenhouse at 25 °C under natural light to allow germination and growth. There were 16 replicates of each of the treated and control plants arranged in a randomized block design.

2.2.3. Nut inoculation using dry conidia

Surface sterilized nuts were coated with *B. bassiana* strain GHA by shaking them with dry conidia powder in a 200 ml plastic cup on a shaker (Fisher Scientific, Waltham, MA) at 80 rpm for 10 min, according to Cherry et al. (2004). Nuts were planted as described above, with 16 replicates of each of the treated and control plants arranged in a randomized block design.

2.2.4. Nut inoculation by soil drenching

Inoculation followed a procedure adapted from Greenfield et al.

(2016). A total of 16 nuts for each of the treatment and control were planted in pots as described above. Each pot was drenched with 300 ml of *B. bassiana* strain GHA ($1x10^8$ conidia ml^{-1} suspension containing 0.05% Silwet L-77) applied to the soil surface at 7, 14, or 21 days post-planting. Control plant pots were inoculated with 100 ml of sterile distilled water containing 0.05% Silwet L-77. The plants were arranged in the greenhouse in a randomized block design.

2.3. Assessment of colonization in plants inoculated with B. bassiana by re-isolation and in vitro culturing

Sixty days post inoculation, four pecan seedlings from each treatment and the controls were sampled for assessment. Plant were asymptomatic, and leaves, stems and roots were sampled on each replicate plant. Colonization was assessed according to Posada et al. (2007). Plant parts were washed in running tap water and sectioned into small pieces (ca. 3 cm) using a sterile scalpel. Under the laminar flow hood, leaves, stems and roots were surface-sterilized separately by immersion in 0.5% sodium hypochlorite for 2 min, followed by immersion in 70% ethanol for 2 min (Arnold et al., 2003) and rinsing in sterile distilled water three times. Samples were surface-dried on sterile paper towel. The cut ends were trimmed off, and the samples were further cut into CA. 4 mm pieces. Five pieces were plated on PDA (Difco, Sparks, MD) and also plated on Doberski and Tribe medium (Doberski and Tribe, 1980). Cultures were incubated at 25 °C for 21 days in the dark. Colonization was assessed on every plant by presence or absence of typical mycelial growth of *B. bassiana*.

2.4. Molecular assessment of plant colonization by B. bassiana

2.4.1. DNA extraction from plant samples and fungal re-isolates

Samples of the pecan plant tissues were collected from a selection of the pecan seedlings as described above. The plant samples, together with corresponding *B. bassiana* cultures on PDA (re-isolated from the different plant parts) were subjected to molecular diagnosis to confirm the identity of *B. bassiana*. DNA extracted from the commercial *B. bassiana* strain GHA grown on PDA was used as a positive control. Plant sample material (100–200 mg) was finely cut and placed in 2 ml microfuge tubes and stored at −80 °C overnight. A Genogrinder (Thomas Scientific, Swedesboro, NJ) was used to grind samples at 1750 rpm for three minutes. DNA was extracted from the sample using a ZR Fungal/Bacterial DNA MinPrep Kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. A 100–200 mg sample was used to isolate DNA of the corresponding in vitro cultured isolates and the commercial product culture. Sterile distilled water was used as a blank control to check for contamination in the samples. The DNA samples were stored at −20 °C until they were processed for PCR.

2.4.2. PCR amplification

Beauveria bassiana DNA was detected using a PCR Phire kit (Thermo Fisher Scientific, Waltham, MA) and following the protocol described by Landa et al. (2013). Oligonucleotide primer pairs ITS1F (5′ *CTTGG TCATTTAGAGGAAGTAA*3′) (Gardes and Bruns, 1993) and ITS4 (5′ *TCCTCCGCTTATTGATATGC* 3′) (White et al., 1990) were used for the first stage amplification and primers BB.fw (5′ *GAACCTACCTATCGTT GCTTC* 3′) and BB.rv (5′ *ATTCGAGGTCAACGTTCAG* 3′) (Landa et al., 2013) were used for the second stage amplification. PCR products were run on an agarose gel, stained with gel red (Biotium, Inc., Fremont, CA) and viewed under UV light. An All-Purpose Hi-Lo DNA Marker (Bionexus, Oakland CA) was used as a marker to visually estimate amplicon size. Samples with amplicons of the correct size were sequenced at the Genomics Core Laboratory of the University of Kentucky Chandler Hospital (Lexington, KY) using Sanger sequencing and the resulting sequence aligned using the BLAST tool on GenBank (National Center for Biotechnology Institute, National Institute for Health, Bethesda, MD) to confirm identity. The chromatographs were viewed and edited as needed using Chromas (Technelysium Pty Ltd, QLD, Australia).

2.5. Pathogenicity of the re-isolated fungus against insect pests

2.5.1. Koch's postulates

Fungal colonies obtained from re-isolated *B. bassiana* strain GHA from inoculated plant material were subcultured onto Doberski and Tribe medium to satisfy the re-isolation stage of Koch's postulates (Vega et al., 2012), and for subsequent DNA extraction for molecular confirmation of re-isolation. Individual cultures were established and grown for 14 days at 25 °C in the dark. Two model susceptible host insects were used to verify pathogenicity of the re-isolated fungus: the yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), and the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). *Beauveria bassiana* strain GHA was cultured under the same conditions for comparison with the endophytic *B. bassiana*. Larvae (10) of *T. molitor* were released onto the PDA plates containing the colonies of *B. bassiana* and allowed free movement for 15 min to ensure inoculation with the fungus. Larvae were removed and incubated in 90 mm Petri dishes lined with moistened Whatman No. 1 filter paper (Fisher Scientific, Waltham, MA) at 25 °C for seven days. The same procedure was followed for bioassays using larvae of *G. mellonella* purchased from Vanderhorst Wholesale, Inc. (St. Marys, OH). Controls consisted of larvae placed on PDA before incubating them as described above. There were five replicates and the experiment was repeated once using a different batch of fungal inoculum. Mortality of the larvae was recorded seven days post inoculation.

2.5.2. Pathogenicity against the pecan weevil

This bioassay was conducted to establish whether *C. caryae* (target pest) is susceptible to re-isolated *B. bassiana* parasitism. *C. caryae* larvae were allowed to burrow individually in 20 g of autoclaved, oven dried loamy sand soil brought to 16% field capacity in 50 ml plastic cups. The sand was moistened with 2.2 ml water, and inoculated separately with 1 ml of 4×10^6 conidial suspensions (in sterile distilled water containing 0.05% Silwet L-77) obtained from cultures of *B. bassiana* from either the plant material, from *G. mellonella* cadavers, or from *B. bassiana* strain GHA (prepared as described previously) and incubated at 25 °C for 21 days in the dark. The experiment was repeated (Trial 2) with different batches of fungal inoculum, with four and three replicates (seven larvae per replicate) in Trial 1 and Trial 2, respectively. Larval mortality was assessed 21 days post-inoculation.

2.6. Leaf-disc assay of endophytic B. bassiana against two pecan aphid species

Two trials were conducted to determine whether endophytic colonization of pecan seedlings by *B. bassiana* resulted in reduced populations of pecan aphids. Trials were conducted based on Shapiro-Ilan et al. (2008). The first trial was performed using *M. caryaefoliae* and the second trial using *M. caryella*. Prior to the test, all the plants chosen for the endophytic treatment were confirmed to still have endophytic *B. bassiana* presence in leaves based on the procedures described above in Section 2.3. Pecan (cv. Desirable) seedlings inoculated using the drench method and the dry method were included in each test. Plants were chosen randomly based on availability. Once the plant was positive for endophytic presence we did not consider the original method of inoculation to be of consequence; indeed, the lack of effects of inoculation method on virulence was confirmed in an earlier part of our study (see Results Section 3.3.). The seedlings were inoculated in the summer of 2017, retained in a pecan orchard at Byron, GA until October 2018, when they were returned to the greenhouse.

Five pecan leaf discs (2 cm diam) from each inoculation method or the control plants (without endophytic *B. bassiana*) were placed in 100 mm diam Petri dishes half-filled with 1.5% sterile water agar. Two aphids per leaf disc, ten aphids per plate were added. The plates were

incubated under natural light at room temperature (ca. 22 C ± 1 °C). After 5 days, the number of surviving aphids was recorded. In each trial (i.e., different aphid species), there were 12 replicate seedlings for each method of inoculation (drench and dry) and the controls.

2.7. Statistical analysis

To test for homogeneity of the inoculation method (control, drench, dry or soak) versus plant part (root, stem or leaf), scores (0 = absent or 1 = present) were summarized in a frequency table. Pearson's Chi-Square (Lombard et al., 2011) and Monte Carlo's exact test (Besag and Clifford, 1989) were used for testing of independence.

The results from the Koch's postulates tests and the pecan weevil study were subjected to an analysis of variance (ANOVA) with fungal inoculum source as the main factor. The standardized residuals were normally distributed (Shapiro-Wilk test) and therefore the means of the significant mortalities were separated using Fisher's unprotected *t*-test (least significant difference; LSD) at $\alpha = 0.05$ (Snedecor and Cochran, 1980).

The effect of endophytic colonization method (drench vs. dry) of pecan by *B. bassiana* for the trials with the two pecan aphid species (*M. caryaefoliae* and *M. caryella*) was first explored using a two-way ANOVA to determine whether there were differences due to inoculation method within aphid species (P = 0.1747 and 0.6799, respectively). As there was no effect of inoculation methods, data were combined for analysis (within aphid species). The number of surviving aphids was compared between endophyte seedlings and the control using t-tests. Based on residual plots, data were square-root transformed prior to analysis; models were chosen based on goodness of fit and residual diagnostics (Southwood, 1978; Steel and Torrie, 1980). All analyses were performed using SAS Version 9.4 (SAS Institute Inc., 2016).

3. Results

3.1. Assessment of colonization in plants inoculated with B. bassiana by re-isolation and in vitro culturing

The three different inoculation methods all resulted in seedlings that tested positive for endophytic *B. bassiana* (Figs. 1 and 2). The fungus was recovered in either leaves, stems or roots. While a significant difference (*Chi-Square value = 20.4; df = 3; P = 0.0001*) was observed in terms of inoculation method (wet seed soak = 50 ± 27%, dry coating = 16 ± 9% and soil drench = 45 ± 21%), there was no significant difference (*Chi-Square value = 2.825; df = 6; P = 0.830*) for the recovery of the fungus from different plant parts (Leaf = 50 ± 20%, stem = 34 ± 12% and roots = 29 ± 8%). There was no recovery of *B. bassiana* in any of the control plants.

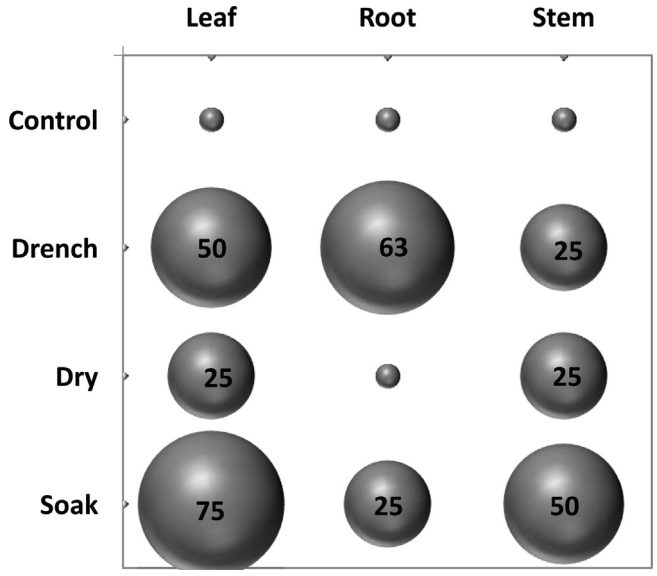

Fig. 1. Mean plant colonization representing by cumulative number of positive samples from different tissue types (roots, stem and leaves) per inoculation method. The numbers are mean percentage colonization (small bubble = 0) post-inoculation with *Beauveria bassiana* strain GHA using three different inoculation methods (wet seed soak, dry coating and soil drench).

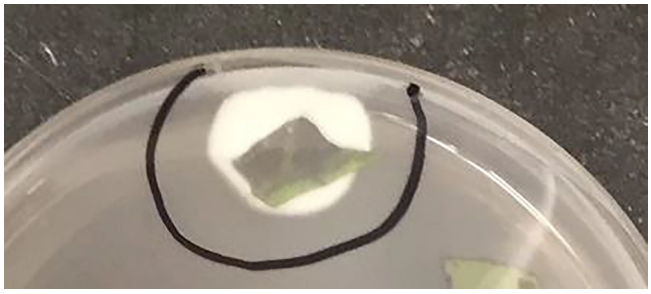

Fig. 2. An example of endophytic *Beauveria bassiana* growing out of plant tissue from a pecan seedling (specifically from a portion of a surface-sterilized leaf). The medium for fungal growth is potato dextrose agar.

3.2. Molecular assessment of plant colonization by B. bassiana

The PCR amplification was consistent in generating an amplicon of the appropriate size (464 bp), specific for *B. bassiana* in all five repeats only for the positive control. The sample of pith from the plants receiving the drench treatment also produced an amplicon indicating presence of *B. bassiana*. No other samples produced an amplicon of the appropriate size. Sequencing confirmed the identity of the amplified fragment in the positive control and the sample of pith from the drench treated plant materials as *B. bassiana*. Alignment against previously characterized sequence of *B. bassiana* on GenBank was 100% (against accession MH483713.1). Samples which received inoculation by drenching or by dry inoculation produced amplicons unreliably, but of approximately the appropriate size (441 bp). Based on sequencing, they were identified as *Fusarium equiseti* (Corda) Sacc. (Ascomycota: Hypocreales) with 99% homology (against accession MF380754.1). All samples isolated from the various plant parts and grown on PDA were confirmed as *B. bassiana* based on amplicon size.

3.3. Pathogenicity of the re-isolated fungus against insect pests

3.3.1. Koch's postulates

Re-isolated samples from endophytically colonized seedlings using the three inoculation methods were confirmed to be pathogenic to both *T. molitor* and *G. mellonella*. There were no significant differences in virulence of the re-isolated fungi (Soak, Dry and Drench treatments) against the two species (Fig. 3). However, percentage mortality of the endophytic *B. bassiana* was significantly lower (*F = 13.0; df = 4; P < 0.0001*) when compared to that of *B. bassiana* strain GHA when using *T. molitor* in the bioassay. With *G. mellonella*, there were no differences in virulence among isolates obtained from the seedlings inoculated using the three different methods, or when comparing the re-isolated strain with *B. bassiana* strain GHA, with mortality consistently > 60%. However, control mortality was significantly lower than all the treatments (*F = 14.9; df = 4; P < 0.0001*).

3.3.2. Virulence against the pecan weevil

Endophytic *B. bassiana* re-isolated from the plant material was pathogenic against *C. caryae* (Fig. 4). Mean percentage mortality of *C. caryae* as a result of infection from the re-isolated endophytic *B. bassiana* vs. that produced in *G. mellonella* infected hosts was comparable but differed significantly from the mortality in the control

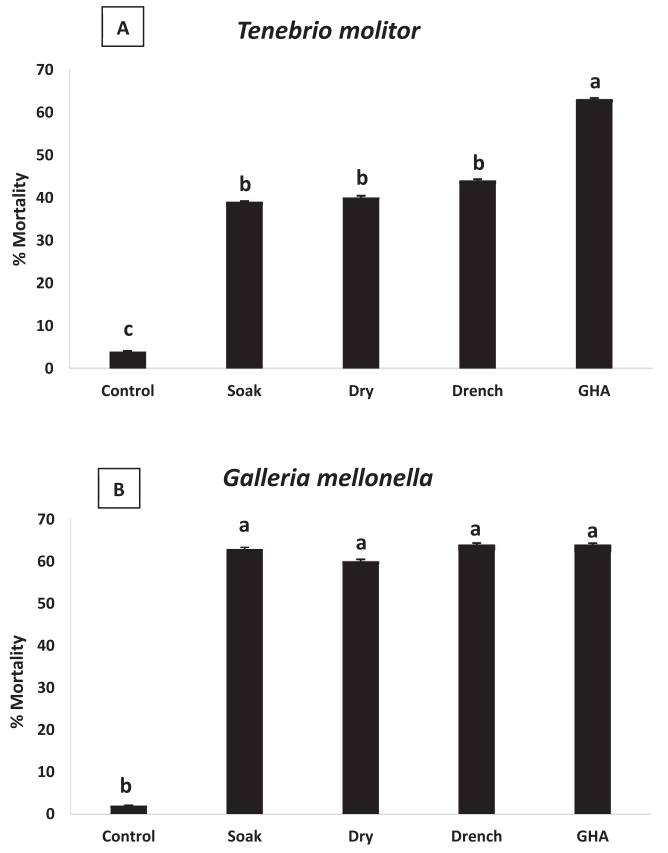

Fig. 3. Virulence comparison of *Beauveria bassiana* strain GHA with endophytic isolates of *B. bassiana* re-isolated from colonized plants and screened against *Tenebrio molitor* (A) and *Galleria mellonella* (B) seven days post-inoculation under laboratory conditions. Standard error bars are indicated. Means with the same lowercase letter indicate no significant difference based on an unprotected Fisher's LSD ($\alpha = 0.05$).

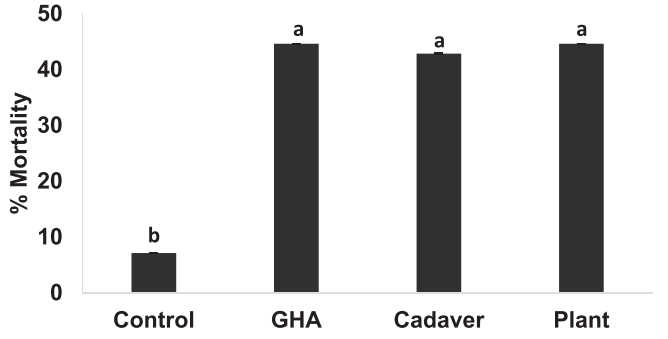

Fig. 4. Mean percentage mortality of *Curculio caryae* 21 days post-inoculation with (1) a commercial product-derived strain (GHA) of *Beauveria bassiana*, (2) an isolate from a GHA-infected cadaver of *Galleria mellonella* and (3) an isolate of *B. bassiana* (GHA) growing endophytically in pecans. Standard error bars are indicated. Means with the same lowercase letter indicate no significant difference based on an unprotected Fisher's LSD ($\alpha = 0.05$).

($F = 4.9$; $df = 3$; $P = 0.003$). Virulence as measured by percentage mortality for both plant and insect derived inoculum were similar to the commercial *B. bassiana* strain GHA.

3.4. Leaf-disc assay of endophytic B. bassiana against two pecan aphid species

Populations of both aphid species were reduced significantly as a result of being placed on leaf discs from pecan seedlings endophytically

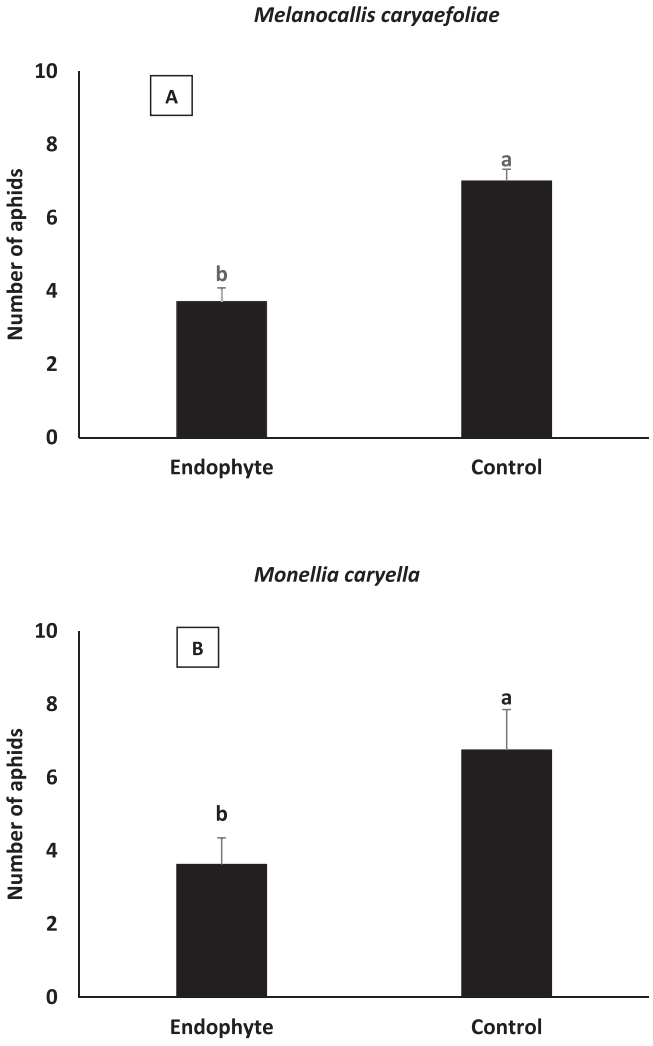

Fig. 5. Cumulative mean survival of the aphid species (A) *Melanocallis caryaefoliae* and (B) *Monellia caryella* five days post-incubation following exposure (for feeding) to pecan leaf discs colonized by endophytic *Beauveria bassiana*. Standard error bars are indicated. Means with the same lowercase letter indicate no significant difference based on *t*-test.

colonized with *B. bassiana* (Fig. 5). Mean survival of aphids in treatments was significantly lower when compared to survival of aphids placed on control leaf discs (from seedlings not inoculated with *B. bassiana*) ($T = -6.2$; $df = 33.3$; $P < 0.0001$ for *M. caryaefoliae,* and $T = -2.5$; $df = 10$; $P = 0.03$ for *M. caryella*).

4. Discussion

We have demonstrated that endophytic colonization of pecan seedlings with *B. bassiana* strain GHA is possible using artificial inoculation. Compared to herbaceous annuals, reports of fungal entomopathogen endophytism in woody perennials is uncommon. Fungal entomopathogen endophytism has been reported in American hornbeam (Bills and Polishook, 1991), cacao (Posada and Vega, 2005), date palm (Gómez-Vidal et al., 2006, 2009), coffee (Posada et al., 2007), pine (Reay et al., 2010; Brownbridge et al., 2012; Lefort et al., 2016), grapevine (Jaber, 2015; Rondot and Reineke, 2018), rooibos (*Aspalathus linearis*; Hatting, 2017) and horse-chestnut (Barta, 2018).

Although inoculation was restricted to seeds and roots in our study, *B. bassiana* endophytism was confirmed in surface-sterilized roots, stems and leaves. Fungal re-isolation from plant tissue samples distal to the point of inoculation (*e.g.*, seed) is probably an indication of vertical

transmission within the host (Shahzad et al., 2018; Vega 2018). This phenomenon has been reported, not only in annuals like opium poppy (*Papaver somniferum*, Quesada-Moraga et al., 2014) and wheat (*Triticum aestivum*, Sánchez-Rodríguez et al., 2018), but also in the woody perennial *Pinus radiata* (Lefort et al., 2016). *Beauveria* spp. are natural endophytes in *Pinus* spp. (Ganley and Newcombe, 2006; Reay et al., 2010), yet such association is still to be confirmed in pecan. From an IPM perspective, however, endophytic establishment of *B. bassiana* by means of seed soaking (yielding 50% recovery in our study) seems feasible. This notion is supported by Brownbridge et al. (2012), reporting successful inoculation of *B. bassiana* into *P. radiata* via seed coating or root dipping.

The successful establishment of *B. bassiana* strain GHA in pecan seedlings was confirmed by bioassay (Koch's postulates) and molecular methods. The amplicons produced using the diagnostic primers indicated that *B. bassiana* had become endophytic, which was confirmed by sequence identity. This further supports the results from culturing surface sterilized plant material, from which *B. bassiana* strain GHA was re-isolated and we concluded that it was growing endophytically in pecan. Furthermore, those samples isolated from the plant and grown on PDA (and completing Koch's postulates) were confirmed as *B. bassiana*, with the amplicon size confirming the identity. However, detection of the amplicon was not entirely consistent for all samples, although sequencing did confirm that *B. bassiana* was present in at least some of these samples. The molecular tests did not detect *B. bassiana* in the roots, even though the fungus was isolated from roots when grown on PDA, fulfilling Koch's postulates. Roots may contain a very low titer of *B. bassiana* that precluded detection. Furthermore, pecan contains many secondary compounds that could inhibit PCR reactions, or at least affect their reliability. Previous work has acknowledged the inhibitory effects of secondary compounds on PCR in some plant species (Healey et al., 2014), and pecan is known to contain various phenolics and tannins (Diehl et al., 1992). Furthermore, the concentration of *B. bassiana* DNA might be expected to be very low in direct extractions from the pecan host tissue. This would further reduce the likelihood of reliable amplification. Previous reports have based identification of endophytic *B. bassiana* on recovery of the strain using fungus isolated from plant material and grown on media (Larran et al., 2002; Posada and Vega, 2005; Posada et al., 2007; Vega et al., 2008; Jaber and Enkerli, 2016). To overcome the problem of potential interference with potentially inhibitory compounds in the roots, the pure, isolated fungal colonies were used to confirm that the fungus that was isolated from the roots was *B. bassiana* based on amplicon size. All isolates were positive.

An amplicon of very similar size to that of *B. bassiana* was observed in some samples. Upon sequencing, it was determined to be *F. equiseti*. The amplicon could easily be confused with that of *B. bassiana*. The confirmation of DNA sequence of *F. equiseti* in some of these samples bears out the report of this fungus associated with pecan seed in Brazil and to be pathogenic to pecan seedlings (Lazarotto et al., 2014). It is also noteworthy as it is a species of *Fusarium* known to produce mycotoxins, which might contaminate pecan nutmeats.

On re-isolation of the fungus, all three inoculation methods yielded cultures that produced conidia that were pathogenic to the three insect species screened (*C. caryae, G. mellonella* and *T. molitor*). For *T. molitor*, virulence of the re-isolated fungus appeared lower than in the original population that did not pass through the plant, yet no differences were detected for the other insect hosts. This indicates that virulence of endophytic fungi relative to the original population may vary across hosts (indeed preliminary data also shows that the virulence of plant-passaged fungi can increase relative to the original population, unpublished data). Importantly, passaging-through and re-isolation of *B. bassiana* strain GHA from an insect versus a plant host, did not affect virulence to a target pest, *C. caryae* larvae. Our results concur not only with those of Barta (2018), but also with data against another curculionid, the banana weevil *Cosmopolites sordidus* (Coleoptera: Curculionidae) (Akello et al., 2010). These authors reported no differences in colony appearance, growth rate or virulence between endophytic and wild-type isolates of *B. bassiana* strain G41. Although *in planta* tests against nut-feeding larvae of *C. caryae* have not yet been performed, further research on vertical translocation of *B. bassiana* in pecan is warranted.

Endophyte activity against sap-feeding aphids has also been reported in various crops (Gurulingappa et al., 2010; Akello and Sikora, 2012; Castillo Lopez et al., 2014). Survival of both aphid species, *M. caryaefoliae* and *M. caryella*, was reduced when exposed to leaf discs of pecan from seedlings endophytically colonized with *B. bassiana*. Based on this observation, we contend that pecan seedlings or mature trees colonized endophytically with *B. bassiana* may have reduced populations of aphids, as well as other insect species associated with the tree (e.g., *C. caryae* and stink bugs). Although pecan cultivation in South Africa remains largely unchallenged by the principal (exotic) pecan pests occurring in the US (Hatting et al., 2019a; Hatting, 2018), endophytic protection against the yellow pecan aphid, fig-tree borer, *Phryneta spinator* (Coleoptera: Cerambycidae), chafer, *Anomala ustulata* (Coleoptera: Scarabaeidae: Rutelinae), mottled stink bug, *Pseudatelus raptorius* (Hemiptera: Pentatomidae) and/or African bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), is being explored (Hatting, unpublished data); with at least five commercial products available on the local market (Hatting et al., 2019b). In addition to suppressing insect pests, endophytic *B. bassiana* is known to suppress plant pathogens, including *Pythium* spp. causing damping-off in cotton (Griffin et al., 2005; Griffin, 2007), *Rhizoctonia solani* causing damping- off in tomato (Ownley et al., 2008), zucchini yellow mosaic virus in squash (Jaber and Salem, 2014) and *Plasmopara viticola* causing downy mildew in grapevines (Jaber, 2015). Therefore, it is conceivable that endophytic *B. bassiana* will provide benefits reducing severity of fungal diseases of pecan, including *Venturia effusa* (pecan scab), *Glomerella cingulata* (anthracnose) and *Phytophthora* spp.

Pecan trees are established in the field as seedlings, so endophytes could be introduced prior to planting. If an established endophyte can persist in pecan trees without loss of activity, endophytes may provide protection against insect pests and plant diseases for extended periods given the lifespan of a pecan tree is ca. 50 years (Payne, 2005). To date we have documented endophytic activity in pecan seedlings for > 1.5 years (Shapiro, unpublished data).

5. Conclusions

To our knowledge, this is the first report of successful inoculation of a fungal entomopathogen as an endophyte in pecan seedlings. Moreover, our trials demonstrated that endophytic *B. bassiana*, re-isolated from the host plant, retained its pathogenicity against the pecan weevil, *C. caryae*. Similarly, endophytically-colonized leaves had a negative impact on the survival of two aphid species (*M. caryaefoliae* and *M. caryella*). Future studies will focus on tracking persistence of the endophyte over time and determining other benefits in insect pest and plant disease management, as well as the impact on tree growth.

6. Author Statement

All authors provided a significant contribution to the conception, design, execution, or interpretation of the reported study.

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or proprietary product is solely for the purpose of providing specific information and does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2019.104102.

References

Akello, J., Sikora, R., 2012. Systemic acropedal influence of endophyte seed treatment on *Acyrthosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. Biol. Control 61, 215–221.

Akello, J., Dubois, T., Coyne, D., Kyamanywa, S., 2010. The pathogenicity of *Beauveria bassiana*: what happens after an endophytic phase in plants? Commun. Agric. Appl. Biol. Sci. 75, 273–278.

Arnold, A.E., Mejía, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., Herre, E.A., 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc. Natl. Acad. Sci. U.S.A. 100, 15649–15654.

Barta, M., 2018. *In planta* bioassay on the effects of endophytic *Beauveria* strains against larvae of horse-chestnut leaf miner (*Cameraria ohridella*). Biol. Control 121, 88–98.

Besag, J., Clifford, P., 1989. Generalized Monte Carlo significance tests. Biometrika 76, 633–642.

Bills, G.F., Polishook, J.D., 1991. Microfungi from *Carpinus caroliniana*. Can. J. Bot. 69, 1477–1482.

Brownbridge, M., Reay, S.D., Nelson, T.L., Glare, T.R., 2012. Persistence of *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte following inoculation of radiata pine seed and seedlings. Biol. Control 61, 194–200.

Castillo Lopez, D., Sword, G.A., 2015. The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (*Gossypium hirsutum*) and negatively affect survival of the cotton bollworm (*Helicoverpa zea*). Biol. Control 89, 53–60.

Castillo Lopez, D., Zhu-Salzman, K., Ek-Ramos, M.J., Sword, G.A., 2014. The entomopathogenic fungal endophytes *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) and *Beauveria bassiana* negatively affect cotton aphid reproduction under both greenhouse and field conditions. PLoS ONE 9 (8), e103891.

Cherry, A.J., Banito, A., Djegui, D., Lomer, C., 2004. Suppression of the stem-borer *Sesamia calamistis* (Lepidoptera; Noctuidae) in maize following seed dressing, topical application and stem injection with African isolates of *Beauveria bassiana*. Int. J. Pest Manage. 50, 67–73.

de Faria, M.R., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control 43, 237–256.

Diehl, S.V., Graves Jr., C.H., Hedin, P.A., 1992. Cytochemical responses of pecan to *Cladosporium caryigenum*: development of specific histological indicators to identify and analyze in situ fungitoxic phenols. Phytopathology 82, 1033–1036.

Doberski, J.W., Tribe, H.T., 1980. Isolation of entomogenous fungi from elm bark and soil with reference to ecology of *Beauveria bassiana* and *Metarhizium anisopliae*. Trans. Br. Mycol. Soc. 74, 95–100.

Dutcher, J.D., 1991. Insects and pecan production. In: Wood, B.W., Payne, J.A. (Eds.), Pecan Husbandry Challenges and Opportunities. National Technical Information Service, Springfield, VA, pp. 43–46.

Ganley, J.R., Newcombe, G., 2006. Fungal endophytes in seeds and needles of *Pinus monticola*. Mycol. Res. 110, 318–327.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.

Goettel, M.S., Inglis, G.D., 1997. Fungi: hyphomycetes. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, pp. 213–249.

Gómez-Vidal, S., Lopez-Llorca, L.V., Jansson, H.B., Salinas, J., 2006. Endophytic colonization of date palm (*Phoenix dactylifera* L.) leaves by entomopathogenic fungi. Micron 37, 624–632.

Gómez-Vidal, S., Salinas, J., Tena, M., Lopez-Llorca, L.V., 2009. Proteomic analysis of date palm (*Phoenix dactylifera* L.) responses to endophytic colonization by entomopathogenic fungi. Electrophoresis 30, 2996–3005.

Greenfield, M., Gómez-Jiménez, M.I., Ortiz, V., Vega, F.E., Kramer, M., Parsa, S., 2016. *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation. Biol. Control 95, 40–48.

Griffin, M.R., 2007. Beauveria bassiana, a cotton endophyte with biocontrol activity against seedling disease. PhD Dissertation, University of Tennessee, Knoxville, USA. 163 pp.

Griffin, M., Ownley, B., Klingeman, W., Pereira, R., 2005. Biocontrol of *Rhizoctonia* damping-off of cotton with endophytic *Beauveria bassiana*. Phytopathology 95 (6), S36.

Gurulingappa, P., Sword, G.A., Murdoch, G., McGee, P.A., 2010. Colonization of crop plants by fungal entomopathogens and their effects on two insect pests when *in planta*. Biol. Control 55, 34–41.

Hatting, J.L., 2017. Major insect pests and their natural enemies associated with cultivation of Rooibos, *Aspalathus linearis* (Burm. f.) R. Dahlgren, in South Africa: a review. S. Afr. J. Bot. 110, 118–123.

Hatting, J.L., 2018. Insects associated with pecan cultivation in South Africa. South African Pecan Nut Producers Association NPC (SAPPA). ISBN: 978-0-621-46904-2.

Hatting, J.L., Calitz, F.J., Du Toit, H., Germishuizen, B., 2019a. Effect of broad-spectrum insecticide applications on insect pest damage and nut mass of pecan, Carya illinoinensis (Juglandaceae), in the Vaalharts production region of South Africa. Int. J. Trop. Insect Sci. 39, 219–227.

Hatting, J.L., Moore, S.D., Malan, A.P., 2019b. Microbial control of phytophagous invertebrate pests in South Africa: current status and future prospects. J. Invertebr. Pathol. 165, 54–66.

Healey, A., Furtado, A., Cooper, T., Henry, R.J., 2014. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. Plant Meth. 10, 21.

Jaber, L.R., 2015. Grapevine leaf tissue colonization by the fungal entomopathogen *Beauveria bassiana s.l.* and its effect against downy mildew. BioControl 60, 103–112.

Jaber, L.R., Salem, N.M., 2014. Endophytic colonization of squash by the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) for managing zucchini yellow mosaic virus in cucurbits. Biocontrol Sci. Technol. 24, 1096–1109.

Jaber, L.R., Enkerli, J., 2016. Effect of seed treatment duration on growth and colonization of *Vicia faba* by endophytic *Beauveria bassiana* and *Metarhizium brunneum*. Biol. Control 103, 187–195.

Jaber, R.L., Ownley, B., 2018. Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? Biol. Control 116, 36–45.

Jallow, M.F.A., Dugassa-Gobena, D., Vidal, S., 2004. Indirect interaction between an unspecialized endophytic fungus and a polyphagous moth. Basic Appl. Ecol. 5, 183–191.

Jallow, M.F.A., Dugassa-Gobena, D., Vidal, S., 2008. Influence of an endophytic fungus on host plant selection by a polyphagous moth via volatile spectrum changes. Arthropod-Plant Interact. 2, 53–62.

Kim, Y., Dharmasena, S., 2018. Price discovery and integration in U.S. pecan markets. J. Food Distrib. Res. 49, 39–47.

Lacey, L.A., Georgis, R., 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. J. Nematol. 44, 218–225.

Landa, B.B., López-Diaz, C., Jiménez-Fernández, D., Montes-Borrego, M., Muñoz-Ledesma, F.J., Ortiz-Urquiza, A., Quesada-Moraga, E., 2013. *In-planta* detection and monitorization of endophytic colonization by a *Beauveria bassiana* strain using a new-developed nested and quantitative PCR-based assay and confocal laser scanning microscopy. J. Invertebr. Pathol. 114, 128–138.

Larran, S., Perelló, A., Simón, M.R., Moreno, V., 2002. Isolation and analysis of endophytic microorganisms in wheat (*Triticum aestivum* L.) leaves. World J. Microbiol. Biotechnol. 18, 683–686.

Lazarotto, M., Muniz, M.F.B., dos Santos, R.F., Blume, E., Harakawa, R., Hamann, F.A., 2014. First report of *Fusarium equiseti* associated on pecan (*Carya illinoinensis*) seeds in Brazil. Plant Dis. 98, 847.

Lefort, M.-C., McKinnon, A.C., Nelson, T.L., Glare, T.R., 2016. Natural occurrence of the entomopathogenic fungi *Beauveria bassiana* as a vertically transmitted endophyte of *Pinus radiata* and its effect on above- and below-ground insect pests. N. Z. Plant Prot. 69, 68–77.

Lombard, C., van der Merwe, L., Kele, T., Mouton, S., 2011. Elementary Statistics for Business and Economics. Pearson Education South-Africa (Pty), pp. 352.

Ownley, B.H., Griffin, M.R., Klingeman, W.E., Gwinn, K.D., Moulton, J.K., Pereira, R.M., 2008. *Beauveria bassiana*: endophytic colonization and plant disease control. J. Invertebr. Pathol. 98, 267–270.

Parsa, S., García-Lemos, A.M., Castillo, K., Ortiz, V., López-Lavalle, L.A.B., Braun, J., Vega, F.E., 2016. Fungal endophytes in germinated seeds of the common bean, *Phaseolus vulgaris*. Fungal Biol. 120, 783–790.

Parsa, S., Ortiz, V., Vega, F.E., 2013. Establishing fungal entomopathogens as endophytes: towards endophytic biological control. J. Vis. Exp. 74, e50360.

Payne, T.J., 2005. Nuts. In: Barrett, D.M., Somogyi, L., Ramaswamy, H. (Eds.), Processing Fruits: Science and Technology, second ed. CRC Press LLC, Boca Raton, pp. 766–808.

Posada, F., Vega, F.E., 2005. Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*). Mycologia 97, 1195–1200.

Posada, F., Aime, M.C., Peterson, S.W., Rehner, S.A., Vega, F.E., 2007. Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). Mycol. Res. 111, 748–757.

Quesada-Moraga, E., López-Díaz, C., Landa, B.B., 2014. The hidden habit of the entomopathogenic fungus *Beauveria bassiana*: first demonstration of vertical plant transmission. PLoS ONE 9, e89278.

Reay, S.D., Brownbridge, M., Gicquel, B., Cummings, N.J., Nelson, T.L., 2010. Isolation and characterization of endophytic *Beauveria* spp. (Ascomycota: Hypocreales) from *Pinus radiata* in New Zealand forests. Biol. Control 54, 52–60.

Rondot, Y., Reineke, A., 2018. Endophytic *Beauveria bassiana* in grapevine *Vitis vinifera* (L.) reduces infestation with piercing-sucking insects. Biol. Control 116, 82–89.

Sánchez-Rodríguez, A.R., Raya-Díaz, S., Zamarreño, A.M., García-Mina, J.M., Del Campillo, M.C., Quesada-Moraga, E., 2018. An endophytic *Beauveria bassiana* strain increases spike production in bread and durum wheat plants and effectively controls cotton leafworm (*Spodoptera littoralis*) larvae. Biol. Control 116, 90–102.

SAS Institute Inc., 2016. SAS/STAT User's Guide Version 9.4, SAS Institute, Cary, NC, USA.

Shahzad, R., Khan, A., Bilal, S., Asaf, S., Lee, I., 2018. What is there in seeds? Vertically transmitted endophytic resources for sustainable improvement in plant growth. Front. Plant Sci. 9, 24.

Shapiro-Ilan, D.I., Cottrell, T.E., Jackson, M.A., Wood, B.W., 2013. Control of key pecan insect pests using biorational pesticides. J. Econ. Entomol. 106, 257–266.

Shapiro-Ilan, D.I., Cottrell, T.E., Jackson, M.A., Wooda, B.C., 2008. Virulence of Hypocreales fungi to pecan aphids (Hemiptera: Aphididae) in the laboratory. J.

Invertebr. Pathol. 99, 312–317.
Skinner, M., Parker, B.L., Kim, J.S., 2014. Role of entomopathogenic fungi in integrated pest management. In: Abrol, D.P. (Ed.), Integrated Pest Management: Current Concepts and Ecological Perspectives. Academic Press, San Diego, pp. 169–191.
Smith, G.S., O'Day, M.H., Reid, W., 2019. Pecan Pest Management: Insects and Diseases. Department of Entomology, Kansas State University. (https://extension2.missouri.edu/mp711), Accessed: 2018/02/287.
Snedecor, G.W., Cochran, W.G., 1980. Statistical Methods, 7th ed. Iowa State University Press, Ames.
Southwood, T.R.E., 1978. Ecological Methods: With Particular Reference to the Study of Insect Populations. Chapman and Hall, London.
Steel, R.G.D., Torrie, J.H., 1980. Principles and Procedures of Statistics. McGraw-Hill Book Company, New York, NY.
Vega, F.E., 2018. The use of fungal entomopathogens as endophytes in biological control: a review. Mycologia 110, 4–30.
Vega, F.E., Posada, F., Aime, M.C., Pava-Ripoll, M., Infante, F., Rehner, S.A., 2008. Entomopathogenic fungal endophytes. Biol. Control 46, 72–82.
Vega, F.E., Meyling, N.V., Luangsa-ard, J.J., Blackwell, M., 2012. Fungal entomopathogens. In: Vega, F.E., Kaya, H.K. (Eds.), Insect Pathology, second ed. Academic Press, San Diego, pp. 171–220.
Vincent, C., Goettel, M.S., Lazarovits, G. (Eds.), 2007. Biological Control: A Global Perspective. CAB International/AAFC, Wallingford, United Kingdom.
White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.
Wilson, D., 1995. Endophyte: the evolution of a term, and clarification of its use and definition. Oikos 73, 274–276.
Wraight, S.P., Inglis, G.D., Goettel, M.S., 2007. Fungi. In: Lacey, L.A., Kaya, H.K. (Eds.), Field Manual of Techniques in Invertebrate Pathology: Application and Evaluation of Pathogens for Control of Insects and Other Invertebrate Pest, 2nd ed. Springer, Dordrecht, The Netherlands.