Initial Studies on Beneficial Fungi that Can Live Inside Pecan Trees and Provide Protection from Insects and Disease

David Shapiro-Ilan[a] Tshimangadzo Ramakuwela[b], Clive Bock[a], Justin Hatting[b], Fernando E. Vega[c], Lenny Wells[d], George N. Mbata[e]

[a]*USDA-ARS, South Eastern Fruit and Tree Nut Research Laboratory, Byron, GA* 31008, *USA*

[b]*Agricultural Research Council-Small Grains (ARC-SG), Bethlehem, South Africa*

[c] *Sustainable Perennial Crops Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705, USA*

[d]*Department of Horticulture, University of Georgia, 4604 Research Way, Tifton, GA 31793, USA*

[e]*Department of Biology, Fort Valley State University, Fort Valley, GA 31030,* USA

Corresponding author E-mail address: David.Shapiro@ars.usda.gov

Summary

Entomopathogenic (aka beneficial) fungi are commercially available biopesticides that are used to control a wide variety of economically important insect pests in various cropping systems. Some of these fungi, such as *Beauveria bassiana*, can kill pecan pests such as pecan weevil and pecan aphids. When used commercially, the beneficial fungi are generally applied to the crop using standard spray equipment. However, certain beneficial fungi, such as *B. bassiana,* have also been found to kill insect pests when living inside plants as endophytes. An endophyte is a microorganism (often fungi or bacteria) that lives inside a plant without causing disease. Conceivably, if endophytic fungi can protect a plant from insects or disease, then treatment costs may be lower compared with spray programs. In this research, we discovered that two beneficial fungal species (*B. bassiana* and *Metarhizium brunneum*) can be inoculated into pecan tree seedlings and exist as endophytes. Moreover, we discovered that the established endophytic fungi can provide protection to pecan. In laboratory and greenhouse tests we observed reduced survival in pecan aphids and reduced growth of the pecan disease,

Phytophthora shuck and kernel rot. In future research we will expand our studies to determine the potential for the endophyte approach under field conditions.

Introduction

Pecan productivity is limited by various insect pests and diseases. Some of the economically important pecan insect pests include pecan weevil (*Curculio caryae*), aphids [black pecan aphid (*Melanocallis caryaefoliae*), blackmargined aphid (*Monellia caryella*), and yellow pecan aphid, (*Monelliopsis pecanis*)], and stink bugs. Pecan diseases include scab (*Venturia effusa*), anthracnose (*Glomerella cirngulata*) and *Phytophthora* spp.

Generally, insect pests and disease are controlled by chemical pesticides (Hudson and Acebes, 2019). Due to environmental and regulatory concerns, research toward developing alternative control measures is warranted. In Southeastern pecan orchards, species of entomopathogenic (insect-killing) fungi occur naturally, such as the white muscardine fungus, *Beauveria bassiana* (Figure 1) and the green muscardine fungus, *Metarhizium brunneum* (Figure 2). The beneficial fungus *Beauveria bassiana* is particularly widespread (Shapiro-Ilan et al., 2003). This fungus can provide up to 50% natural mortality in pecan weevil just based on native fungus already living in the orchard. Additionally, the fungus can kill higher levels of pecan weevil (up to 80%) when commercial formulations are applied via spray equipment to the pecan trunk or to the soil (Shapiro-Ilan et al., 2017). Both fungi (*B. bassiana* and *M. brunneum*) can also kill pecan aphids (Shapiro-Ilan et al., 2008).

A drawback to using entomopathogenic fungi, such as *B. bassiana* and *M. brunneum* as biopesticides, is that spray applications can be costly relative to many chemical insecticides. Another option to achieve pest control using entomopathogenic fungi may be establishing the beneficial fungi as endophytes in the crop plant. An endophyte is a microorganism (often fungi or bacteria) that lives inside a plant without causing disease. Insect killing fungi, such as *B. bassiana,* have been found to exist as endophytes in various crops such as corn, bananas, beans, cacao, cotton, coffee, pine, tomato, and wheat. Endophytic insect-killing fungi have been shown to control various insect pests including aphids in cotton and weevils in banana. Also, insect-killing fungi such as *B. bassiana*, when established as endophytes, have been shown to reduce certain plant diseases such as those in the genera *Fusarium* and *Pythium.* Prior to our research, it was not known if beneficial insect-killing fungi could exist as endophytes in pecan. Our objectives were to 1) determine if entomopathogenic fungi can be established in pecan, and 2) assuming endophytes are established, determine if the fungi provide protection against pecan insect pests and disease. Thus far, most of our work has been on *B. bassiana* but we have also recently initiated research on *M. brunneum*.

Material and Methods

Inoculation of the fungus, B. bassiana, into pecan seeds and seedlings. Pecan seeds were surface sterilized before experimentation. Subsequently, three different methods were implemented to inoculate *B. bassiana* into pecan for endophyte establishment: 1) seed soaking, 2) seed rolling, and 3) seedling drenching. For seed-soaking, the surface sterilized pecan seeds were soaked 24 hours in 300 ml of a suspension containing *B. bassiana* conidia spores (1×10^8 conidia per ml). For the rolling method, seeds were shaken at about 80 rpm in plastic cups with a dry powder of fungal spores. After soaking or rolling, seeds were planted in 6” x 6” pots filled with soil from a nearby pecan orchard. For the drench method, newly planted seeds were drenched with 300 ml of spore suspensions (1×10^8 conidia per ml) 7, 14, or 21 days post-planting.

***Assessment of endophyte status in pecan seedlings*.** Sixty days after treatment, at least four seedlings from each method of inoculation were assessed for the presence of *B. bassiana* fungus as an endophyte within the plant. Cuttings of roots, stems and leaves were surface sterilized and placed on selective agar media in Petri dishes to see if fungus would grow out of the cuttings. The fungus growing out of the plant cuttings were subjected to molecular analysis (DNA extraction and PCR analysis) to verify the fungus was *B. bassiana*.

Additionally, the virulence (killing power) of the fungus re-isolated from inside the plant was verified. Insects used to verify virulence included larvae of the greater wax moth (*Galleria mellonella*), yellow mealworms (*Tenebrio molitor*) and pecan weevil. For wax moth and mealworms, 10 larvae were placed on agar plates that contained a lawn of the fungus obtained from inside pecan seedlings; there were 10 replicate plates per treatment. After 15 minutes of exposure to the fungus, the larvae were removed and placed in Petri dishes with filter paper. Insect larval mortality and signs of fungal infection were recorded one week after exposure. Control larvae were exposed to agar plates without fungus.

For pecan weevil, a soil cup experiment was conducted. Pecan weevil larvae were added to 1 oz. cups containing 20 grams of soil. Fungal spores (4×10^6) that were obtained from fungus re-isolated from pecan seedlings were added to each cup and insect mortality was determined after 21 days. The virulence of the fungus derived from pecan seedlings was compared to the same number of spores from a commercial formulation of *B. bassiana* and a control (no fungus) was also included. There were four replicates of seven larvae per treatment and the experiment was then repeated with three replicates per treatment.

Greenhouse experiments to determine the impact of endophytic B. bassiana on pecan aphids. Two experiments were conducted: one with black pecan aphids and the second with blackmargined aphids. Twelve pecan seedlings confirmed to have endophytic *B. bassiana* and twelve seedlings without the fungus (control plants) were randomly selected for each experiment. Five leaves were removed from each seedling and placed on agar dishes. Ten aphids were placed in each dish. The number of surviving aphids was recorded after five days.

Experiments to determine if endophytic B. bassiana can suppress Phytophthora shuck and kernel rot. Methods used were based on those described by Shapiro-Ilan et al. (2014). Briefly, detached pecan leaves from endophyte positive seedlings and endophyte negative seedlings (controls) were added to 150 cm Petri dishes containing 3% agar. The endophytic leaves were taken from two-year old Desirable seedlings that were inoculated with *B. bassiana* fungus; control leaves were from Desirable seedlings of an equal age that were not exposed to the fungus. A plug of *Phytophthora cactorum* was added to each leaf. The size of *Phytophthora* lesion was measured after one week. There were five replicate leaves for the control and treatment.

Experiments to determine if the green muscardine fungus M. brunneum, can exist as an endophyte in pecan. Using the same approaches as described above (soaking seeds, rolling seeds in powder, and drenching seedlings), pecan was exposed to the fungus, *M. brunneum.* Additionally, another method of endophyte inoculation was attempted – foliar spray. In this approach, the leaves of seedlings were sprayed with a fungal suspension containing spores. The foliar approach, if successful, may be a relatively easy way to inoculate pecan with endophytic fungi. After exposure to the fungus, re-isolation of fungi from the plant and subsequent confirmation via virulence assays were conducted as described above.

Results

***Assessment of B. bassiana endophyte status in pecan seedlings*.** All methods of fungal inoculation (seed soaking, rolling seeds in dry power, and drenching seedlings) resulted in *B. bassiana* establishment as an endophyte (Figure 3) (Ramakuwela et al., 2019). In terms of endophyte establishment, the drench or soak methods were superior to the dry-rolling method of inoculation. The *B. bassiana* fungus was detected in leaves, stems and roots of pecan seedlings. Fungal presence was verified by molecular methods as well as in virulence tests (Ramakuwela et al., 2019). For wax moth and pecan weevil larvae, no significant differences in insect mortality were observed when comparing the *B. bassiana* fungus that was re-isolated from pecan seedlings versus an equal number of spores from the commercial *B. bassiana* formulation (insect mortality ranged from 40% to 60%), whereas for mealworms, the level of mortality was higher in the commercial fungus treatment relative to the fungus derived from the plant (60% versus 40%, respectively).

Greenhouse experiments to determine the impact of endophytic B. bassiana on pecan aphids. Populations of both black pecan aphids and blackmargined aphids were reduced when exposed to leaf discs from endophytic *B. bassiana* pecan seedlings compared to control leaves (without *B. bassiana* fungus) (Figure 4).

Experiments to determine if endophytic B. bassiana can suppress Phytophthora shuck and kernel rot. *Phytophthora* lesion size was significantly reduced in leaves containing endophytic *B. bassiana* compared to control leaves (without the endophytic fungus) (Figure 5).

Experiments to determine if the green muscardine fungus, M. brunneum, can exist as an endophyte in pecan. *Metarhizium brunneum* was also established as an endophyte in pecan seedlings using the seedling drench or seed soaking approaches. The fungus was also established as an endophyte in pecan seedlings using the foliar spray method. Endophytic *M. brunneum* was successfully established in the following cultivars: Caddo, Desirable, Elliot, and Pawnee.

Conclusions and Discussion

- An insect-killing fungus, *Beauveria bassiana*, was successfully introduced as an endophyte into pecan seedlings.
- Our work represents the first successful inoculation of a fungal entomopathogen as an endophyte in pecan seedlings.
- The presence of the endophytic fungus was verified in pecan via molecular methods and virulence experiments against insects (Ramakuwela et al., 2019).
- So far, endophytic *B. bassiana* has been documented to remain in pecan seedlings up to two years (and still counting).
- In greenhouse/lab tests, seedling leaves with endophytic *B. bassiana* caused reduced populations of black pecan aphids and blackmargined aphids compared with seedlings that did not contain the fungus.
- Moreover, in greenhouse/lab tests, seedling leaves with endophytic *B. bassiana* caused reduced growth of *Phytophthora* lesions compared with seedlings that did not contain the fungus.
- Another entomopathogenic fungus, *Metarhizium brunneum*, was also found to be amenable to inoculation into pecan seedlings and establishment as an endophyte. This fungus was also inoculated into pecan via foliar sprays.

- To date, the following cultivars have been shown to be amenable to the endophyte approach: Avalon, Caddo, Desirable, Elliot, Pawnee.

Future Research.

- Compare the amenability of endophyte inoculation among pecan cultivars. We have thus far shown that a wide variety of pecan cultivars are amenable to the endophyte approach; in research currently underway we are comparing which cultivars may be the most susceptible to inoculation.
- Compare the impact of the *M. brunneum* fungus to the *B. bassiana* fungus for ability to exist as an endophyte in pecan and provide protection to the tree.
- Determine if mature pecan trees can be inoculated to establish fungal endophytes via drenching or foliar sprays (preliminary evidence shows this is probable).
- Expand efficacy tests on endophyte seedlings and trees in the greenhouse and under field conditions to measure the level of protection from pecan insect pests and disease.
- Measure yield and other physiological effects of endophytic fungi on pecan. Some studies on other crops indicate that entomopathogenic fungal endophytes enhance plant growth and health (Dara, 2019).

Acknowledgements

We appreciate technical assistance from Kate Anderson, Stacy Byrd and Minling Zhang. We thank the Georgia Commodity Commission for partial funding of this research. Support to T. Ramakuwela was provided by the USDA-Foreign Agriculture Service - Borlaug International Agricultural Science and Technology Fellowship Program. This article reports the results of research only. Mention of a trademark 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.

References

Dara, S.K. 2019. Non-Entomopathogenic roles of entomopathogenic fungi in promoting plant health and growth. Insects 10, 277.

Hudson, W. and A. Acebes, 2019. Commercial pecan insect control (bearing trees), pp. 1-5. *In* L. Wells [ed.] 2019 Commercial Pecan Spray Guide. University of Georgia Extension Bulletin 841.

Ramakuwela, T., Hatting, J., Bock, C., Vega, F.E., Wells, L., Mbata, G.N., Shapiro-Ilan, D.I. 2019. Establishment of *Beauveria bassiana a*s a fungal endophyte in pecan (*Carya illinoinensis*) seedlings and its virulence against pecan insect pests. Biological Control 104, 104102

Shapiro-Ilan, D. I., W. Gardner, J. R. Fuxa, B. W. Wood, K. Nguyen, B. Adams, R. A. Humber, and M. J. Hall. 2003. Survey of entomopathogenic nematodes and fungi endemic to pecan orchards of the southeastern US and their virulence to the pecan weevil (Coleoptera: Curculionidae). Environmental Entomology 32: 187-195.

Shapiro-Ilan, D. I., T. E. Cottrell, M. A. Jackson, and B. W. Wood. 2008. Virulence of Hypocreales fungi to pecan aphids (Hemiptera: Aphididae) in the laboratory. Journal of Invertebrate Pathology 99, 312-317.

Shapiro-Ilan, D.I., Bock, C.H., Hotchkiss, M.W. 2014. Suppression of pecan and peach pathogens on different substrates using *Xenorhabdus bovienii* and *Photorhabdus luminescens*. Biological Control 77, 1–6.

Shapiro-Ilan, D.I., T. E. Cottrell, C. Bock, K. Mai, D. Boykin, L. Wells, W. G. Hudson, and R. F. Mizell III. 2017. Control of pecan weevil with microbial biopesticides. Environmental Entomology 46, 1299–1304.

Figure 1. The white muscardine fungus, *Beauveria bassiana*, infecting a pecan weevil adult (photo taken by Louis Tedders, USDA-ARS).

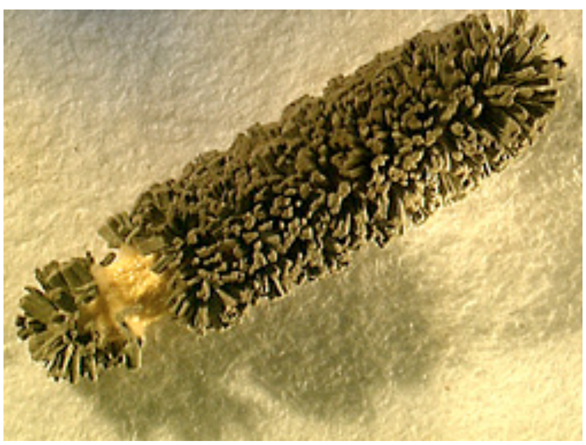


Figure 2. The green muscardine fungus, *Metarhizium brunneum*, growing out of a root maggot (photo credit Stefan Jaronski, USDA-ARS).

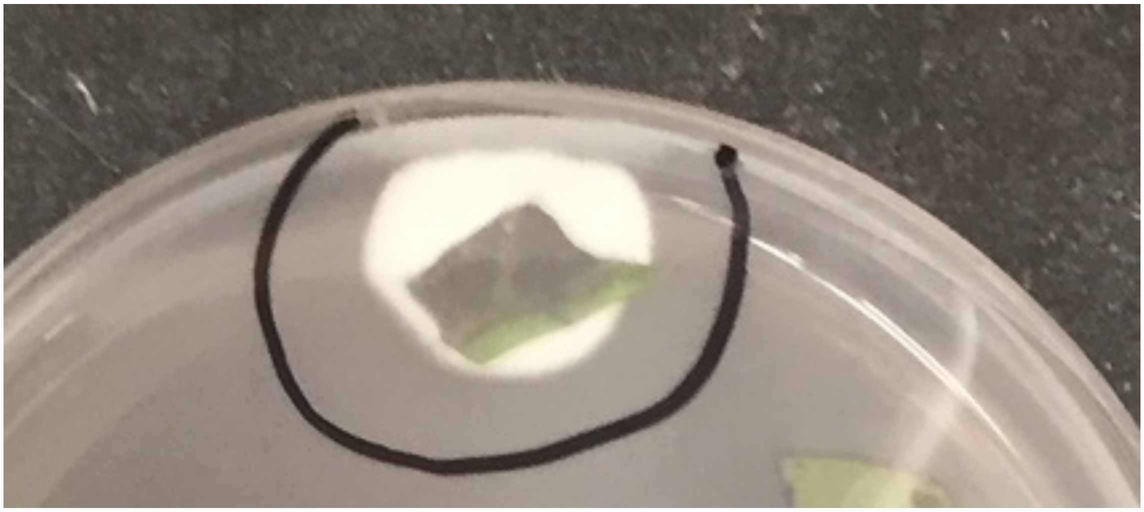
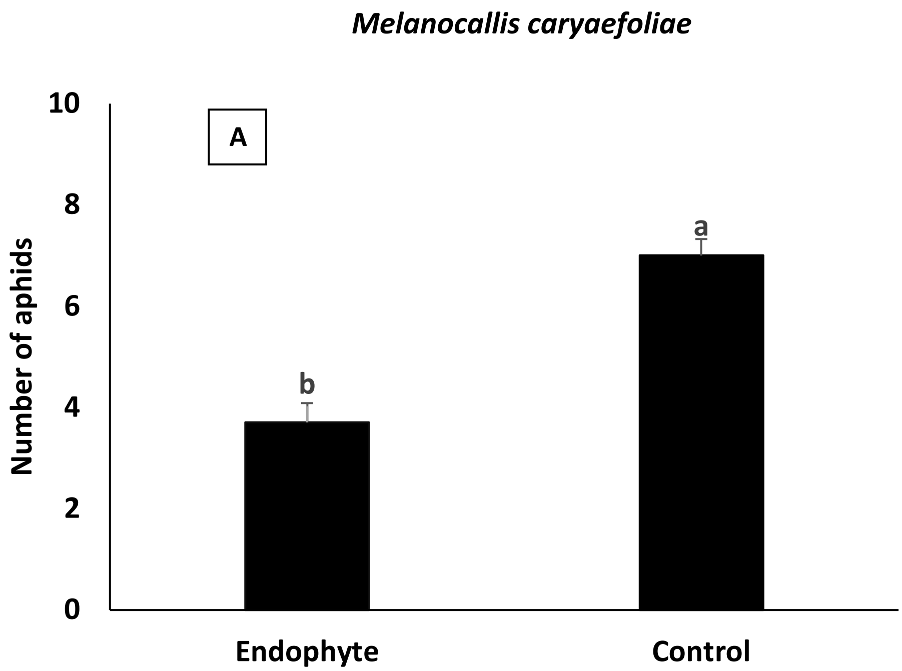


Figure. 3. An example of endophytic *Beauveria bassiana* growing out of plant tissue from a pecan seedling (specifically from a portion of a surface-sterilized leaf). The leaf cutting is on a nutritive agar plate



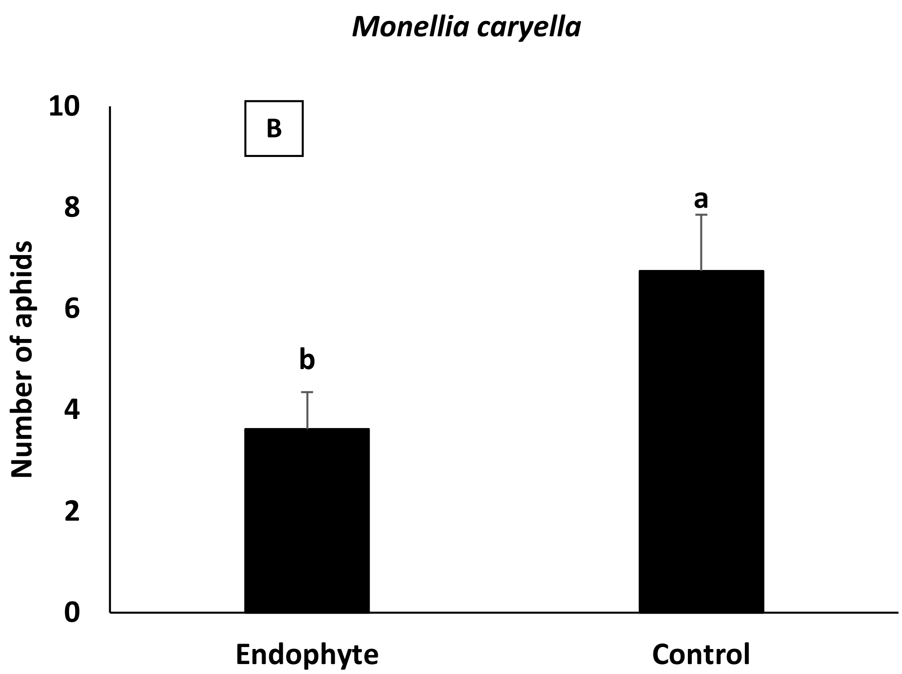


Figure 4. Survival of **(A)** black pecan aphids (*Melanocallis caryaefoliae*) and **(B)** blackmargined aphids (*Monellia caryella*) 5 days after exposure to pecan leaves with endophytic fungus (*Beauveria bassiana*) or without fungus (control).

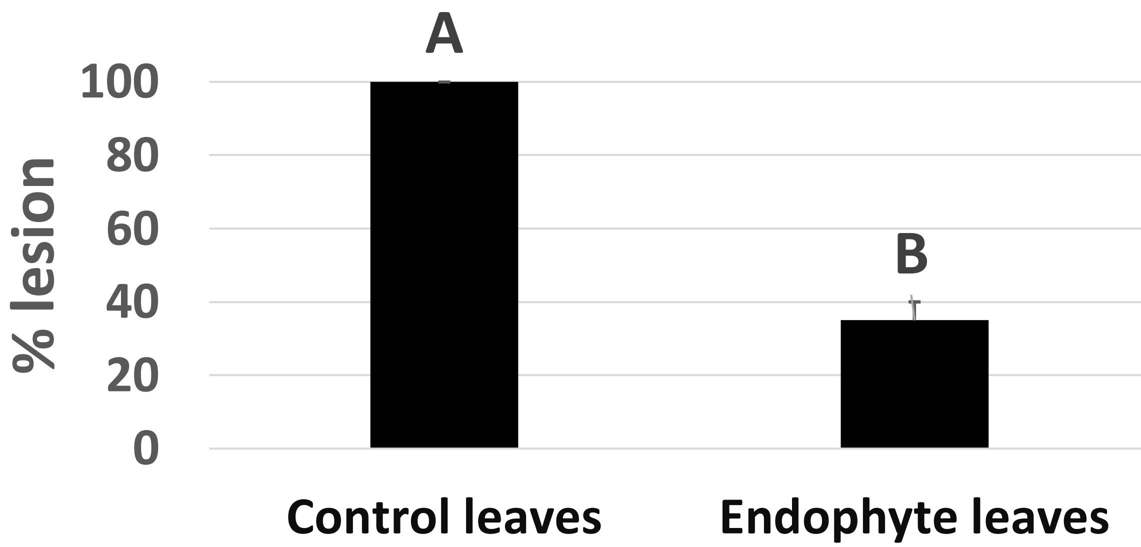


Figure 5. Percentage *Phytophthora cactorum* lesions on pecan leaves containing endophytic fungus (*Beauveria bassiana*) versus leaves without the fungus (control).