Virulence of Hypocreales fungi to pecan aphids (Hemiptera: Aphididae) in the laboratory

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A B S T R A C T

There is need for efficacious biocontrol agents for aphids in commercial orchards. As a preliminary step to this end we determined the virulence of several Hypocreales fungi to pecan aphids. In the first experiment we tested the virulence of Isaria fumosorosea (ARSEF 3581) blastospores to three pecan aphids Monella caryella, Melanocallis caryaefoliae, and Monelliopsis pecanis under laboratory conditions. Rates of 1 x 10^7 or 1 x 10^8 spores per ml were applied in 2 ml via a spray tower to 90 mm Petri dishes containing 10 aphids each. Mortality and mycosis were determined after 24, 48 and 72 h. Treatment effects were observed by 48 h post-application, and by 72 h the higher application rate caused >90% mortality and mycosis in M. caryella and M. caryaefoliae, whereas <70% was observed in M. pecanis.

We conducted two subsequent experiments (Experiments 2 and 3), using the same methodology, to compare the virulence of several Hypocreales species and strains against the aphid of primary economic concern to most pecan growers, M. caryaefoliae. In Experiment 2, we compared blastospores and conidia of two I. fumosorosea strains (ARSEF 3581 and ATCC 20874 (= strain 97)). The blastospores of ARSEF 3581 and conidia of ATCC 20874 showed higher virulence than other treatments and thus were included in Experiment 3, which also compared the virulence of conidia of Beauveria bassiana (GHA strain) and Metarhizium anisopliae (F52 strain). Results in Experiment 3 indicated the highest virulence in I. fumosorosea 3581 blastospores and M. anisopliae (F52) followed by I. fumosorosea (20874) conidia. The detection of pathogenicity to pecan aphids establishes the potential for commercial usage and additional study. Results reported here will narrow treatments to test in future greenhouse and field trials.

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1. Introduction

Pecan is economically the most important nut crop native to North America (Wood, 2003). The pecan aphid complex consists of three species: the black pecan aphid, Melanocallis caryaefoliae (Davis), the blackmargined aphid, Monella caryella (Fitch), and the yellow pecan aphid, Monelliopsis pecanis Bissell. These aphids feed and reproduce during the foliated growing season, and during early autumn lay eggs that overwinter in bark crevices (Hudson, 2007). All three species are serious pests of pecan because of their potential to reduce current and subsequent season nut yield and quality due to direct and indirect damage that diminish tree energy reserves (Tedders and Wood, 1985; Wood et al., 1987). Generally, M. caryaeefoliae is considered the most economically important aphid in pecans (Cottrell et al., 2002; Wood, 2003).

Current control recommendations for pecan aphids rely on usage of chemical insecticides once populations reach economic thresholds (Dutcher et al., 2003; Hudson et al., 2006). A negative side-effect is that chemical pesticides can lead to pesticide resistance by the aphids and/or the destruction of beneficial natural enemies (Dutcher et al., 2003; Ellington et al., 1995; Pickering et al., 1990), hence, research toward developing alternative pest management tactics is warranted.

Entomopathogenic fungi may have promise as an alternative control measure for aphid management in commercial orchard enterprises. Although certain entomopathogenic fungi, (i.e., several species in the order Entomophthorales) have been reported as natural pathogens that may regulate pecan aphid populations (Ekbo and Pickering, 1990; Pickering et al., 1990), no studies have investigated direct application of fungi to aphids for assessment of inundative or inoculative approaches. Several Hypocreales fungi, such as Beauveria bassiana (Balsamo) Vuillemin, Isaria fumosorosea (Wize), and Metarhizium anisopliae (Metschnikoff) Sorokin have been shown to be virulent to certain aphid species (Mesquita and Vandenberg, 2001; Pell and Vandenberg, 2002; Poprawski et al., 1999; Vandenberg, 1996), as well as to certain other soft bodied Hemiptera (e.g., Wraight et al., 1998, 2000). The objective of this study was to determine the pathogenicity and relative virulence of several Hypocreales fungi to pecan aphids under laboratory conditions.
conditions. Initially, we determined pathogenicity and virulence of a single *I. fumosorosea* strain (ARSEF 3581) to *M. caryaefoliae*, *M. caryella*, and *M. pecans* under laboratory conditions. This strain is being developed as a biocidal agent for various insect pests (Dunlap et al., 2007). Subsequently, focusing on *M. caryaefoliae*, we compared blastospore and conidia virulence of the 3581 strain with that of the commercially available strain *I. fumosorosea* (ATCC 20847), and then compared the most virulent *I. fumosorosea* treatments to conidia of commercially available *B. bassiana* and *M. anisopliae* strains.

2. Materials and methods

*Isaria fumosorosea* ARSEF 3581, *I. fumosorosea* ATCC 20847 (= Strain 97), *B. bassiana* (GHA strain), and *M. anisopliae* (FS2 strain), were used in this study. Blastospores of *I. fumosorosea* NRRL 3581 and ATCC 20847 were produced in a 3 day liquid culture fermentation according to Jackson et al. (2003) using basal media supplemented with 25 g/L acid hydrolyzed casein (Hycase M, Kerry Biosciences, Kerry, Ireland) and with 50 g/L glucose (Difco, Detroit, MI). Briefly, blastospores were harvested by mixing whole cultures with diatomaceous earth, dewatered by vacuum filtration, and air dried to less than 5% moisture as previously described (Jackson et al., 2003). Air dried blastospores were vacuum-packaged and stored up to 44 d at 4 °C prior to rehydration and spray application. Conidia of all fungal isolates tested were produced on Potato Dextrose Agar (PDA) plates incubated at 25 °C, stored up to 16 d at 4 °C, and formulated in water via mechanical suspension (using a micropipette) according to methods described by Goettel and Inglis (1997). Aphids were reared on the foliage of greenhouse-grown pecan seedlings, germinated from open pollinated ‘Curtis’ nuts, at approximately 25 °C and 14:10 (L:D) (Cotrell et al., 2002). All aphids were less than 5 d old when used in experiments.

Virulence assays were conducted based on procedures described by Vandenberg (1996). Blastospores were applied to aphids using an Automatic Potter Spray Tower (Burkard Scientific Limited, Uxbridge, Middx, UK) (Potter, 1952). The spray tower was equipped with an intermediate atomizer and delivered 2 ml of treatment at 69 kpa with an allowance of a 5 s settling period. Prior to the bioassays, spore viability was determined by spraying 2 ml of suspension onto Petri dishes containing 1/4 strength Sabouraud dextrose agar (16.25 g per liter rather than 65 g per liter) with 1% yeast extract (SDAY) and counting the percentage of spores that had germinated after 6 h of incubation at 25 °C (Goettel and Inglis, 1997). Based on three replicate counts the viability estimates ranged from 65.4% (*I. fumosorosea* ATCC 20874 conidia in Experiment 2) to 85.0% (*M. anisopliae* conidia in Experiment 3), the viability estimates were taken into account in calculating application rates of viable spores.

Prior to the bioassays, the actual number of spores deposited for each rate of application was estimated by applying 2 ml of suspension and then counting the number of spores per cm² (at 200× magnification) on two cover slips that were placed on a Petri dish (90 mm) containing 1.5% water-agar, three replicate (disks) per application rate yielded estimates of 5.7 × 10³ and 1.49 × 10⁵ per cm² spores for the 10² and 10⁸ application rates (see below), respectively.

In the first experiment (Experiment 1), we measured the virulence of *I. fumosorosea* ARSEF 3581 blastospores to all three pecan aphid species. Blastospores were applied to 10 alate aphids, which were on five 2.5-cm-diameter leaf discs placed on 1.5% water-agar in a single 90-mm-diameter Petri dish. Two milliliters of *I. fumosorosea* suspension were applied at a rate of 1 × 10⁷ or 1 × 10⁸ viable blastospores per ml. Control dishes received 2 ml of water only. After inoculation the dishes were incubated at 25 °C for 72 h. After 24 h of incubation, insects were moved to fresh leaf discs on Petri dishes without fungus. Mortality was checked daily. Dead insects were removed and placed on 1.5% agar plates at 25 °C for an additional 2 d to check for signs of mycosis. The experiment was set up as a factorial (main effects = fungus rates and aphid species) with four replicates per treatment in a completely randomized design.

In Experiment 2, we compared blastospores and conidial preparations of *I. fumosorosea* ARSEF 3581 and ATCC 20874 for virulence to *M. caryaefoliae*. The procedures were identical to those described above except that mortality was recorded after 24, 48, 72 and 96 h, mycosis was determined after 7 d rather than after 2 d, and the experiment, organized in a completely randomized design, was not analyzed as a factorial (since there was only one aphid species). In Experiment 3, using *M. caryaefoliae* as the target pest, we compared the virulence of blastospores of *I. fumosorosea* ARSEF 3581 and conidia of *I. fumosorosea* ATCC 20874 (because these treatments showed the highest virulence in Experiment 2) with conidia of *B. bassiana* and *M. anisopliae*. The procedures and design in Experiment 3 were identical with those of Experiment 2. All three experiments were repeated once after the first trial was completed.

For the analysis of each experiment, data from both trials were combined, and variation among trials was accounted for as a block effect. Additionally, percentage data were arcsine transformed prior to analysis (SAS, 2001; Steel and Torrie, 1980). Non-transformed means are presented. Treatment differences in aphid mortality (regardless of putative cause) and mycosis (exhibiting signs of fungal infection) were analyzed separately.

In Experiment 1, due to a lack of independence (i.e., a significant interaction reported below), the main effects in the factorial experiment were analyzed separately using ANOVA (Cochran and Cox, 1957; SAS, 2001; Steel and Torrie, 1980). The fungus effect was analyzed within each aphid species by comparing mortality and mycosis observed in the two fungus rates to each other and to the control. The aphid species effect was analyzed for each fungus rate, in this case, however, to avoid potential bias due to unequal control mortality, Abbott’s formula (Abbott, 1925) was applied to the data prior to analysis. In Experiments 2 and 3, two-way ANOVA was applied (SAS, 2001). In all analyses, the Student–Newman–Keuls’ test was used to elucidate treatment effects when a significant F value (P < 0.05) was detected in the ANOVA (SAS, 2001).

3. Results

In Experiment 1, a significant interaction between aphid and fungus effects was detected 3-d post-application in aphid mortality and mycosis (F = 4.21, df = 4, 62, P = 0.0044, and F = 6.75, df = 4, 62, P = 0.0001, respectively). Analysis of the fungus effect on aphid mortality indicated that *I. fumosorosea* (ARSEF 3581) is pathogenic to all three aphid species (Fig. 1). Although no difference in mortality was observed 1-d post-application, the higher application rate of *I. fumosorosea* caused higher aphid mortality than the control by 2-d post-application for *M. caryaefoliae* and by 3-d post-application in the other two species (Table 1, Fig. 1). Three-day post-application mortality in the higher fungal rate reached 100% in *M. caryella* and *M. caryaeoliae*, and ±SE 69.9 ± 5.1% in *M. pecans* (Fig. 1).

Analysis of mycosis by aphid species in Experiment 1 also confirmed pathogenicity. No mycosis was observed 1-d post-application, at 2-d post-application mycosis was observed in the high fungal rate applied to *M. caryella* and *M. pecans*, and at 3-d post-application both application rates of *I. fumosorosea* caused significant mycosis in all species (Table 1, Fig. 2). Also 3-d post-application the higher fungal rate caused greater mycosis than...
the lower rate in all three aphid species (Fig. 2). Mycosis was not observed in control aphids on any of the sample dates (Fig. 2).

When relative fungal susceptibility of aphids was compared, i.e., the analysis of aphid effect, a significant difference in mortality data (after correction with Abbott’s formula) was only detected 3-d post-application. Specifically, at 3-d post-application the $1 \times 10^7$ rate produced higher mortality ($\pm$SE in M. caryella (51.2 $\pm$ 9.1%) than M. caryaeofila (19.6 $\pm$ 7.1%) or M. pecanis (11.7 $\pm$ 7.6%) ($F = 7.45; df = 2, 20; P = 0.004$), and the $1 \times 10^8$ rate produced higher mortality in M. caryella and M. caryaeofila (both 100%) than M. pecanis (62.9 $\pm$ 6.1%) ($F = 46.6; df = 2, 20; P = 0.0001$).

When comparing mycosis among aphid species, a significant difference was only detected at the $1 \times 10^8$ rate 3-d post-application, M. caryella and M. caryaeofila (97.5 $\pm$ 1.6% and 92.5 $\pm$ 3.1%, respectively) exhibited a higher percentage mycosis than M. pecanis (52.9 $\pm$ 6.6%) ($F = 27.3; df = 2, 20; P = 0.0001$). No other differences in aphid susceptibility were detected ($P > 0.05$).

In Experiment 2, which compared blastospores and conidia of I. fumosorosea ARSEF 3581 and ATCC 20874, differences in M. caryaeofila mortality among fungal strains and rates of application were observed (Table 2, Fig. 3). At one day post-inoculation less than 8% M. caryaeofila mortality was observed in all treatments and control (data not shown) and no differences were observed among them (Table 2). Two days post-inoculation at the higher rate ($1 \times 10^8$ spores per ml) ARSEF 3581 and ATCC 20874 blastospores and ATCC 20874 conidia caused higher mortality than 3581 conidia and the control, no differences were observed among treatments or the control at the lower rate (Table 2; Fig. 3). After three days, all treatments (at both rates) caused greater mortality than the control, and all treatments at the higher rate were statistically similar and caused >98% mortality (Table 2; Fig. 3). At the lower rate three days post-inoculation, ARSEF 3581 blastospores caused higher mortality than the other treatments except for ATCC 20874 conidia (Table 2; Fig. 3), thus, these two treatments (ARSEF 3581 blastospores and ATCC conidia) were chosen for further comparison in Experiment 3. Additionally, at the lower rate on day 3, ATCC 20874 conidia caused higher mortality than ARSEF 3581 conidia and similar mortality compared with the blastospore treatments (Table 2; Fig. 3). After four days, all treatments caused >98% mortality (with no difference among them) (Table 2; Fig. 3).

Differences in M. caryaeofila mortality were also observed among treatments in Experiment 3 (Table 2; Fig. 4). One day post-inoculation, M. caryaeofila mortality was <2% in all treatments and the control (data not shown) and no statistical differences in mortality were observed (Table 2). After two days, only three treatments applied at the higher rate exhibited higher mortality than the control, i.e., M. anisopliae (F52), ARSEF 3581, and ATCC 20874 (Table 2; Fig. 4). Additionally, after two days, at the higher rate of application, M. anisopliae conidia and ARSEF 3581 blastospores caused higher mortality than all other treatments, and ATCC 20874 conidia caused higher mortality than B. bassiana (GHA) conidia, no differences were observed at the lower rate (Table 2; Fig. 4). After three days, all fungal treatments exhibited higher mortality than the control, and at the higher rate, M. anisopliae conidia, ARSEF 3581 blastospores, and ATCC 20874 conidia caused higher mortality than B. bassiana conidia, at the lower rate ARSEF 3581 blastospores caused the highest mortality followed by M. anisopliae and ATCC 20874 conidia (which were not different from each other) (Table 2; Fig. 4).

Four days, all treatments exhibited higher mortality than the control, all treatments caused >98% mortality and were not statistically different from each other.

### Table 1

Statistics from laboratory experiments measuring *Isaria fumosorosea* suppression of three pecan aphid species

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Aphid</th>
<th>DAT</th>
<th>$F$</th>
<th>df</th>
<th>$P$</th>
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<td>–</td>
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<td>6.66</td>
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<td>–</td>
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<td>2.10</td>
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<td>63.35</td>
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</table>

a DAT, Days after treatment.

b Zero mycosis was observed in all treatments (hence means squares were zero and $F$ was not calculable).
Statistics from laboratory experiments measuring Hypocreales fungal suppression of Melanocallis caryaefoliae

<table>
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<tr>
<th>Measurement</th>
<th>Experiment</th>
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<th>df</th>
<th>P</th>
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<td>180.71</td>
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</table>

* See text for details on experiments.

b DAT, Days after treatment.

**Fig. 2.** Mean (±SE) percentage of aphids exhibiting overt signs of mycosis one, two, or three d after exposure to Isaria fimicola (ARSEF 3581). Rates of 1 × 10^7 (Low) or 1 × 10^8 (Hi) blastospores per ml were applied in 2 ml via a spray tower to 90 mm Petri dishes containing 10 aphids each. Aphids included blackmargined aphid (Monellia caryella [BMA]), black pecan aphid (Melanocallis caryaefoliae [BPA]), and yellow pecan aphid (Monelliposis pecanis [YPA]). Control = water only. Different letters above bars indicate statistically significant differences within each sample date (SNK test, α = 0.05).

**Fig. 3.** Mean (±SE) mortality of black pecan aphid, Melanocallis caryaefoliae two, three, or four days after treatment (DAT) with blastospores (-B) or conidia (-C) of Isaria fimicola ARSEF 3581 or ATCC 20874. Rates of 1 × 10^7 (Low) or 1 × 10^8 (High) spores per ml were applied in 2 ml via a spray tower to 90 mm Petri dishes containing 10 aphids each. Control = water only. Different letters above bars indicate statistically significant differences within each sample date (SNK test, α = 0.05). Except for the B. bassiana treatment, which caused lower mortality at approximately 80% (Table 2; Fig. 4).

Regarding the percentage of aphids showing signs of mycosis in Experiments 2 and 3, all fungal treated aphids that died exhibited mycosis after seven days, whereas none of the aphids in the control exhibited signs of mycosis. Thus, the analyses for mycosis in Experiments 2 and 3 (Table 2) were similar to the analyses of mortality in terms of statistical separation among treatments. Indeed, the SNK distribution of percentage mycosis was identical to mortality, except in Experiment 2 at three days post-inoculation, mycosis in the lower rate of the ATCC 20847 blastospore treatment was higher than the lower rate of ARSEF 3581 conidia (whereas there was no difference between the two detected in overall mortality, Fig. 3).

**4. Discussion**

Our data indicate that while Isaria fimicola (ARSEF 3581) is pathogenic to all three pecan aphid species tested, M. pecanis is less...
When screening virulence to *M. caryaeformia*, we observed substantial variation among the Hypocreales species and strains tested. Overall, our data provide evidence that the highest virulence was exhibited in *I. fumosorosea* ARSEF 3581 blastospores followed by *M. anisopliae* conidia and then *I. fumosorosea* 20874 conidia with *B. bassiana* (GHA) conidia exhibiting the lowest virulence. In addition to differences among fungal strains or species, we observed varying effects of spore type on virulence within the species *I. fumosorosea*. For ARSEF 3581, blastospores were more virulent than conidia, whereas no significant differences in spore type were detected in ATCC 20874.

Possibly, different culture techniques contributed to the differences we observed across spore types (within or among species and strains). Blastospores were produced in a liquid medium whereas conidia were produced on solid medium; both media were comprised of a different nutritional background. Nutritional content is known to affect the production quality of various Hypocreales species (Shah and Butt, 2005; Vidal et al. 1998). We justified the choice of spore types tested based on what is currently available or being pursued for commercial application. We chose to test only conidia of *B. bassiana* and *M. anisopliae* because conidia are used in the current commercial products and we chose to test both blastospores and conidia for *I. fumosorosea* because active research is currently underway for use of both spore types (Dunlap et al., 2007; Er et al., 2007; Yanagawa et al., 2008). Additionally, we justified our approach and comparisons because we produced the fungal spores using standard methods for each spore type and fungal species in a manner similar to spore type comparisons made in other studies (Behle et al., 2006; Vandenberg et al., 1998). Nonetheless, because culture conditions or media may have contributed to the virulence differences we observed, the results should be viewed in terms of a combination of effects i.e., production factors as well as innate differences among strains, species, or spore types.

The relative virulence among fungal treatments that we observed to *M. caryaeformia* is similar to results reported in some studies that targeted other insects and in contrast to others. Similar to our results, Behle et al. (2006) observed superior virulence in *I. fumosorosea* (ARSEF 3581) blastospores relative to conidia of the same strain when targeting the Mexican bean beetle, *Epilachna varivestis* Mulsant. When targeting the brown citrus aphid, *Toxoptera citricida* (Kirkland), Poprawski et al. (1999) also observed results similar to ours in that conidia of an *M. anisopliae* strain (ARSEF 759) showed higher virulence than conidia of several *I. fumosorosea* strains. In contrast to our results, when targeting the horn fly, *Hematobia irritans* (L.), Lohmeyer and Miller (2006) observed superior virulence in *B. bassiana* (GHA) conidia compared with *I. fumosorosea* strains. Also unlike our results, no virulence differences were observed among blastospores and conidia of three *I. fumosorosea* strains when targeting the Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Vandenberg et al., 1998). Certainly, different results can be expected when targeting different insect hosts.

Our data establish that *I. fumosorosea* is pathogenic to three pecan aphid species and several Hypocreales fungi are pathogenic to *M. caryaeformia* (with varying levels of virulence among the fungi). Our results indicate that research aimed at testing the ability these fungi to suppress pecan aphids under field conditions may be warranted. Achieving efficacy within orchards, however, is likely to be challenging due to environmental conditions that are potentially harmful to the fungus (Goettel and Inglis, 1997; Lacey and Shapiro-Ilan, 2008) and to the likely need for good coverage of the canopy by sprays. Conceivably other strains or species of entomopathogenic fungi are more virulent to pecan aphids or more environmentally tolerant than those we have tested, the potential to identify strains with higher virulence can be explored in additional laboratory and greenhouse experiments.

Fig. 4. Mean (±SE) mortality of black pecan aphid, *Melanococcus caryaeformia* two, three, or four days after treatment (DAT) with *Isaria fumosorosea* ATCC 20874 conidia (I-20874-C). *I. fumosorosea* ARSEF 3581 blastospores (I-3581-B), *Beauveria bassiana* (GHA) strain conidia (Bb-C), or *Metarhizium anisopliae* FS2 strain conidia (Ma-C). Rates of $1 \times 10^7$ (Low) or $1 \times 10^8$ (High) spores per ml were applied in 2 ml via a spray tower to 90 mm Petri dishes aphids each. Control = water only. Different letters above bars indicate statistically significant differences within each sample date (SNK test, $\alpha = 0.05$)
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References


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