Entomopathogenic Fungi as Mortality Factors of Macadamia Felted Coccid, *Eriococcus ironsidei* (Hemiptera: Eriococcidae) in Hawaii

Rosemary Gutierrez-Coarite[1*], Wade P. Heller[2], Mark G. Wright[1], Javier Mollinedo[1], Lisa Keith[3], Lionel Sugiyama[3], and **Stacey Chun[4]**

[1]Entomology Section, Department of Plant and Environmental Protection Sciences, University of Hawaii at Manoa, 3050 Maile Way, Honolulu, HI 96822; [2]Department of Natural Resources and Environmental Management, University of Hawaii at Manoa, 3050 Maile Way, Honolulu, HI 96822; [3]USDA-ARS, Daniel K. Inouye U.S. Pacific Basin Agricultural Research Center, 64 Nowelo St., Hilo, HI 96720; [4]Hawaii Department of Agriculture, 16 E Lanikaula St., Hilo, HI 96720; *Correspondence: gr6@hawaii.edu

Abstract. Entomopathogenic fungi are considered to play a vital role as a biological control agent of many insect populations. Different entomopathogenic fungi were observed infecting *Eriococcus ironsidei* Williams (Hemiptera: Eriococcidae) in a macadamia nut orchard in Honokaa, Hawaii. Here, we report the results of the isolation of the unidentified fungal pathogens observed infecting *E. ironsidei* on macadamia leaves and their identification using molecular techniques. We evaluated the susceptibility of *E. ironsidei* to the isolated fungi and to one commercial formulation of the entomopathogenic fungus, *Beauveria bassiana*. To assess whether any of the isolated pathogens have potential to serve as biocontrol agents, *E. ironsidei* was exposed to isolated fungi. Identified entomopathogens were *Chlorocillium griseum* and *Pleurodesmospora coccorum*. Results of this study confirmed that *C. griseum, P. coccorum,* and *B. bassiana* cause mortality in *E. ironsidei* up to 67%, 78%, and 100%, respectively. The present investigation indicates that *E. ironsidei* is highly susceptible to these fungi and they may have a role in regulating insect pest populations.

Key words: entomopathogens, pathogenicity, *Pleurodesmospora coccorum, Chlorocillium griseum.*

Macadamia felted coccid, *Eriococcus ironsidei* Williams (Hemiptera: Eriococcidae), was first reported from macadamia nut trees (Proteaceae: *Macadamia integrifolia*) in Australia, where it can become a sporadic severe problem on macadamia nut trees (Ironside 1970). *E. ironsidei* was found infecting macadamia trees in South Kona on the island of Hawaii in 2005. By 2009 *E. ironsidei* spread to the east and northern areas of Hawaii island, resulting in severe infestations in many locations (Wright and Conant 2009).

Macadamia felted coccid infests all above-ground parts of trees to feed and reproduce. This insect feeds by inserting its needle-like mouthparts into plant tissue and sucking sap from the plant. Their feeding activity distorts and stunts new growth, causing yellow spotting on older leaves and branch dieback when population densities are high (Jones 2002). On bearing trees, nut yields are reduced and a delay is caused in the drop of mature nuts. This pest can produce several generations throughout the year

and higher populations occur in the drier months. In Australia, insecticidal oils are applied to control this pest, but biological control with predatory insects is effective there; in Hawaii, *E. ironsidei* is usually controlled with insect growth regulators combined with horticultural oils (Wright and Conant 2009). However, widespread and indiscriminate insecticide use could lead to the emergence of pest resistance to these insecticides.

Biological control using microbial pathogens such as entomopathogenic fungi offers a potential alternative to chemical insecticides. Fungal pathogens may be host-specific and nontoxic to nontarget vertebrates (Lacey et al. 2001). Approximately 100,000 species of fungi have been described (Blackwell 2011) placed in over 100 genera, and about 750–1000 are fungal entomopathogens (Roberts and Humber 1981, McCoy et al. 1988, St. Leger and Wang 2010). de Faria and Wraight (2007) identified 171 fungal products used as biocontrol agents since the 1960s, most of them based on the genera *Beauveria, Metarhizium,* and *Isaria*. Isolates of these microbial biocontrol agents are effective against a diversity of insect taxa such as Hemiptera, Coleoptera, Lepidoptera, Thysanoptera, and Orthoptera, which are distributed in approximately 48 families (de Faria and Wraight 2007).

The goal of this study was to identify entomopathogen species naturally infecting *E. ironsidei* in macadamia nut orchards in Hawaii and to confirm their pathogenicity to *E. ironsidei*. Here, we report the results of the isolation and identification of the fungal pathogens infecting *E. ironsidei* on macadamia leaves using molecular techniques. We evaluated the susceptibility of adult and nymphs of *E. ironsidei* to isolated fungi, and to one commercial entomopathogenic fungus, *Beauveria bassiana,* in the laboratory environment, to assess whether isolated fungi could potentially serve as effective biocontrol agents to suppress *E. ironsidei* populations.

Materials and Methods

Fungal isolation. In August 2015, macadamia nut leaves infested with *E. ironsidei* were collected from a macadamia nut orchard (var. 344) in Honokaa, Hawaii (20°03′18″ N, 155°23′43″ W) and brought to the lab for examination. Using dissecting microscopes, conidia from *E. ironsidei* cadavers were transferred to a CTC-4T medium (potato dextrose agar amended with 0.5 g.l⁻¹ chloramphenicol, 0.004 g.l⁻¹ thiabendazole and 0.25 g.l⁻¹ cycloheximide) by plating individual *E. ironsidei* cadavers with whitish mycelium directly onto isolation plates. Plates were incubated at 23°C and 70% relative humidity for 14 days. Following initial isolation, purification of the fungus was done by sub-culturing hyphal tips three times successively using the conventional plate streaking method, to ensure that single genotype cultures were obtained for further identification of the isolates (Tuite 1969).

PCR analysis to identify fungal species. DNA from 14-day-old cultured isolates was extracted using the DNeasy Plant Mini Kit (Qiagen, Germantown MD) and a portion of the ribosomal DNA operon including ITS1, 5.8S, and ITS2 was amplified using fungal-specific primers ITS1 and ITS4 (White 1990) and GoTaq DNA Polymerase (Promega, Madison WI). Cycling parameters included a 2-minute initial denaturation at 95°C, 40 cycles of 30 seconds at each 95°C, 55°C, and 72°C, and a 10-minute final extension at 72°C. PCR products were cleaned up using ExoSAP-It (Thermo Fisher, Waltham MA) and submitted for sequencing by Eurofins Genomics (Louisville KY).

Production of conidial cultures and preparations of conidial suspensions. The fungi collected from the field were

identified as *Pleurodesmospora coccorum* (Hyphomycetes) and *Chlorocillium griseum* (Hypocreales) and were inoculated on potato dextrose agar plates and incubated at 24°C for 14 days. *Beauveria bassiana* strain GHA (BotaniGard®, LAM International Corporation, Butte MT) was used directly from the emulsifiable suspension to create colonies on PDA plates. Ten ml of sterile water containing 0.02% Triton X-100 was added to each plate, and the surface was scraped with a spreader and collected to obtain a concentrated suspension of conidia. The conidial suspension was filtered through cheesecloth to remove hyphal debris, and a Neubauer hemocytometer was used to determine the spore concentrations. Each isolate was adjusted to concentrations of 10 ppm, 30 ppm, and 60 ppm conidia per ml. Conidial suspensions were used immediately for bioassays.

The viability of conidia was determined using the dye exclusion test (Strober, 2001) by mixing 100 µl of conidial suspensions with 100 µl of Trypan Blue stain 0.4% (Thermo Fisher, San Diego CA) and counting live cells with the hemocytometer.

Insects. To collect *E. ironsidei* that were likely not exposed to *C. griseum* and *P. coccorum* in the environment, macadamia leaves infested with *E. ironsidei* were collected from a macadamia orchard in Pahala where the presence of *C. griseum* and *P. coccorum* was not observed. Highly infested leaves were selected (>100 *E. ironsidei* per 6.5 cm^2) for the bioassay experiments.

Fungal conidia and bioassays. Three replicate groups of 10 leaf samples infested with *E. ironsidei* (each sample leaf measured 9.7 cm^2) were immersed in each conidial suspension (conidial viability from 99.7 to 100%) for 10 s. The controls were dipped in sterile 0.02% Triton X-100. The treated infected leaves were then transferred individually to petri dishes

with moist filter paper at the bottom and incubated in the laboratory environment with a relative humidity ranging from 68 to 78% and temperature from 23 to 25°C for 24 days. Reproduction was evaluated by recording daily the numbers of *E. ironsidei* crawlers per sample. The duration that *E. ironsidei* continued to reproduce (reproductive period) in each treatment was also recorded. Mortality due to entomopathogenic fungi was confirmed after the fungal hyphae emerged from the dead *E. ironsidei* crawlers and adults.

Statistical analysis. Data on *E. ironsidei* mortality was converted to a percentage and corrected for mortality of the control (Abbott 1925). Data were analyzed by ANOVA using Genstat (Genstat 2009). For conidial concentrations, treatments were compared using least significant difference comparisons ($P = 0.05$).

Results

In surveys of *E. ironsidei* in Hawaii, fungal mycelium was observed growing on *E. ironsidei* cadavers infesting leaves of macadamia trees (variety 344) in the Hamakua coast of Hawaii island. These macadamia trees were spaced 5.48 m apart in rows 7.62 m apart. Trees were approximately 35 years old with an average plant height of 12 m and with an average canopy diameter of 3.5 m; canopies were overlapping.

Fungal species. The ITS sequences for our *E. ironsidei* entomopathogenic fungi isolates were deposited as GenBank accessions MF581039 (546 bp) and MF581040 (559 bp). To determine the identity of the fungi, these sequences were BLAST queried using the NCBI nr and Mycobank. org (Robert et.al. 2013) databases. Sequence MF581039 was found to be 99% identical to the corresponding region from *Pleurodesmospora coccorum* isolate CBS 458.73, and MF581040 was found to be 99% identical to that from *Chlorocillium*
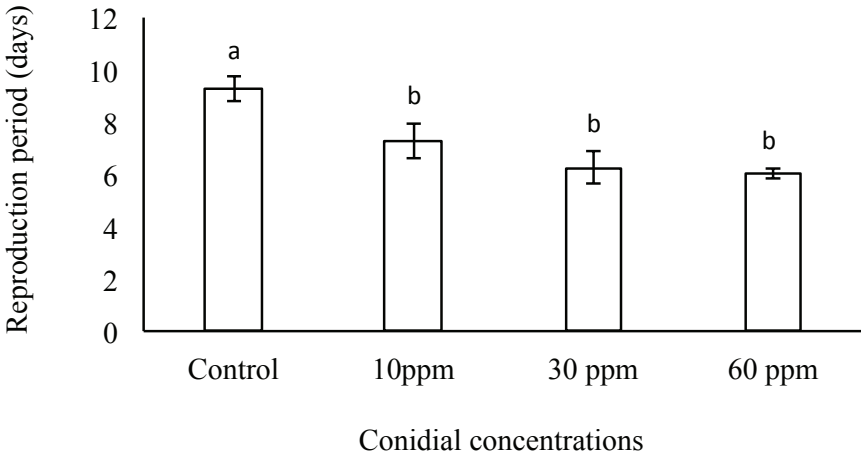
Figure 1. Mean (±SEM) length of time *E. ironsidei* continued to reproduce following treatment with different conidial concentrations of *C. griseum*. Bars with different letters differ significantly (α = 0.05) by LSD.
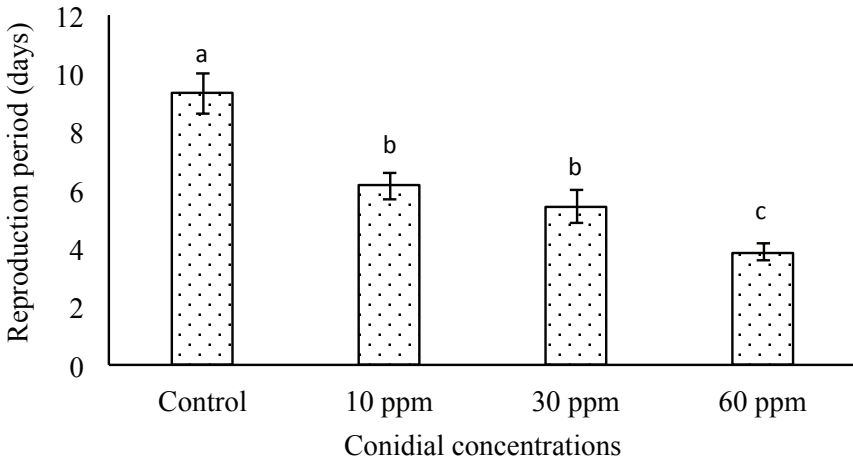

Figure 2. Mean (±SEM) length of time *E. ironsidei* continued to reproduce following treatment with different conidial concentrations of *P. coccorum*. Bars with different letters differ significantly (α = 0.05) by LSD.

griseum isolate ARSEF 7345.

Fungal conidia and bioassays. The reproductive period significantly decreased with increasing conidial concentrations of *C. griseum* (F = 7.70; df = 3, 119; P < 0.0001) (Fig. 1), *P. coccorum* (F = 19.25; df = 3, 119; P < 0.0001) (Fig. 2), and *B. bassiana* (F = 168.15; df = 3, 119; P <

0.0001) as compared to the control (Fig. 3). The reproduction period of *E. ironsidei* was observed to continue up to 9 days in the control treatments; however, the reproduction period decreased to 6 days when this pest was treated with *C. griseum*, to 4 days with *P. coccorum*, and less than one day when treated with *B. bassiana*.
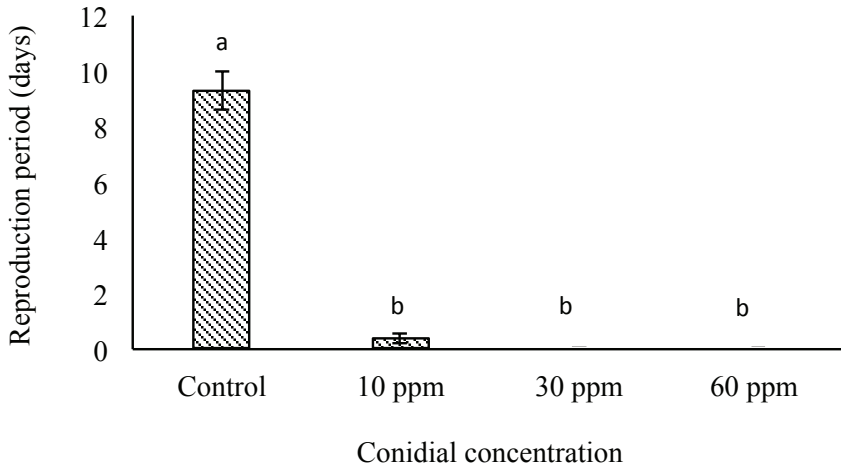
Figure 3. Mean (±SEM) length of time *E. ironsidei* continued to reproduce following treatment with different conidial concentrations of *B. bassiana*. Bars with different letters differ significantly (α = 0.05) by LSD.
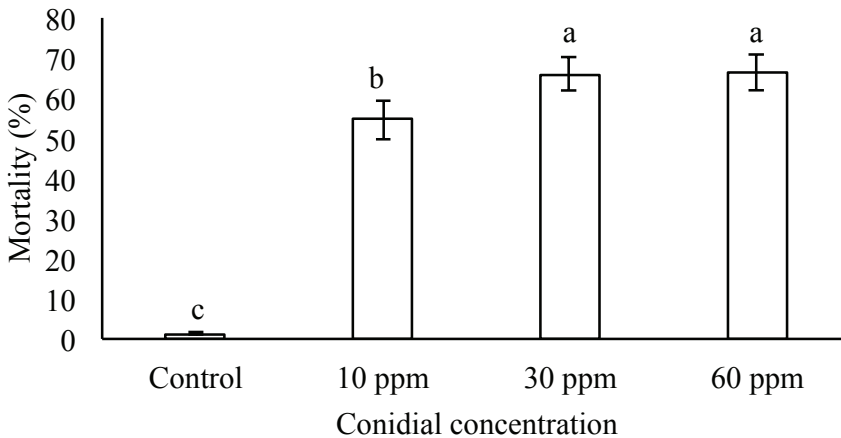

Figure 4. Mean mortality (±SEM) of *E. ironsidei* after dipping in increasing conidial concentrations of *C. griseum*. Bars with different letters differ significantly (α = 0.05) by LSD.

Treatments of fungal concentrations affected the percentage of *E. ironsidei* mortality. Significant differences in mortality were observed when *E. ironsidei* was treated with different conidial concentrations of *C. griseum* (F = 60.81; df = 3, 119; *P* < 0.0001), *P. coccorum* (F = 113.59; df = 3, 119; *P* = 0.0001), and *B. bassiana* (F = 24610.0; df = 3, 119; *P* < 0.0001). *E. ironsidei* mortality caused by *C. griseum* ranged from 55% to 67% (Fig. 4), 67% to 78% by *P. coccorum* (Fig. 5), and 92% to 100% by *B. bassiana* (Fig. 6). *B. bassiana* caused the highest mortality rates compared to *P. coccorum* and *C. griseum*.
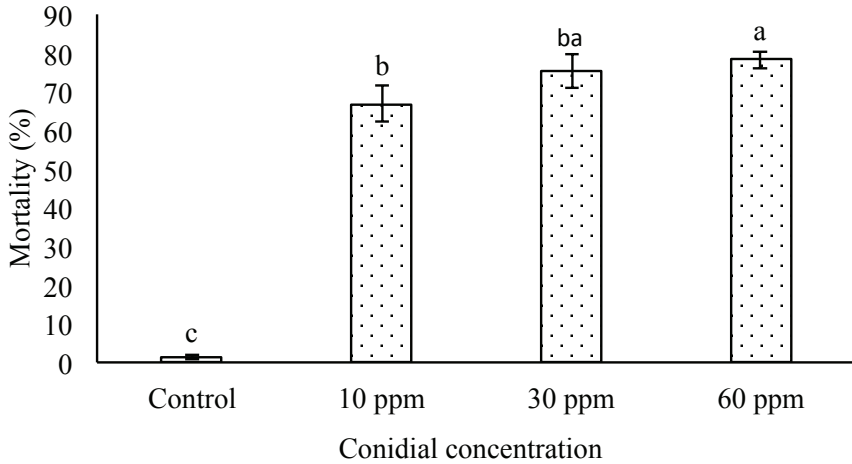
Figure 5. Mean mortality (±SEM) of *E. ironsidei* after dipping in increasing conidial concentrations of *P. coccorum*. Bars with different letters differ significantly (α = 0.05) by LSD.

Discussion

The entomopathogenic fungal species isolated from *E. ironsidei* in the North Hilo District of Hawaii island were identified as *P. coccorum* and *C. griseum*. *P. coccorum* is a common fungus on arthropods, observed by Petch (1931) on *Lecanium* and *Aleyrodes* species, as well as in aphids and leafhoppers. Samson (1980) indicated that *P. coccorum* was observed on various hosts, but most often on Araneidae in samples from Ghana, and it was also found on scale insects from the Galapagos Islands. In a study on pathogens of mites in citrus orchards in Florida, *P. coccorum* was observed infecting the scavenger mite *Tydeus gloveri* Ashmead (Samson and McCoy 1982). However, no studies have been carried out to test the pathogenicity of *P. coccorum*, but its occurrence on non-insect substrates indicates that it may also be saprophytic or mycoparasitic (Samson et al. 1980). *C. griseum* was isolated from spiders and *Pseudococcus* sp. by Petch (1931) in Sri Lanka. This species was also isolated from Coccidae on coffee in Jamaica (Zare 2016).

Results of this study confirmed that *C. griseum*, *P. coccorum*, and *B. bassiana* cause mortality in *E. ironsidei* that is likely dependent on inoculum concentration. Therefore, for the three fungi species, as conidial concentration increased, *E. ironsidei* mortality increased significantly. The highest mortality of *E. ironsidei* was observed at higher conidial concentrations of *B. bassiana*.

Under the experimental laboratory conditions, significant declines in the reproductive period of *E. ironsidei* were observed following contact with *C. griseum*, *B. bassiana*, and *P. coccorum*. *B. bassiana* reduced the reproductive longevity of *E. ironsidei* to the shortest time interval measured, which means that even a low conidial concentration (10^7 conidia.ml^{-1}) may reduce the fecundity of *E. ironsidei*. *Chlorocillium griseum* may take a longer time than *B. bassiana* to begin to reduce *E. ironsidei* longevity (up to 7 days).

Mycelial growth of *B. bassiana* was observed on adults, nymphs, and eggs at the same time; however, the mycelial growth of *P. coccorum* and *C. griseum* occurred
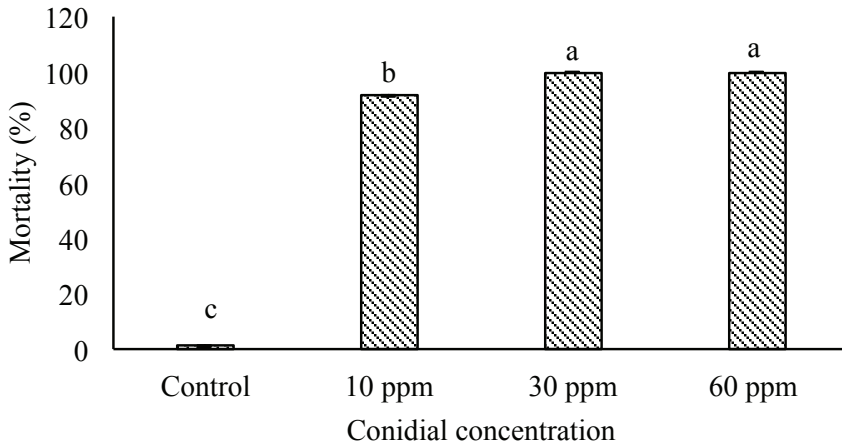
Figure 6. Mean mortality (±SEM) of *E. ironsidei* after dipping in increasing conidial concentrations of *B. bassiana*. Bars with different letters differ significantly (α = 0.05) by LSD.

first on nymphs, later on adults, and finally on eggs of *E. ironsidei*. One possibility is that *P. coccorum* and *C. griseum* more effectively infest soft-bodied life stages, and more time is required to penetrate the shell covering the females to infest the eggs. *Eriococcus ironsidei* eggs continued hatching for a longer time post-treatment, and longevity was longer with *P. coccorum* and *C. griseum* treatments than with *B. bassiana*. Thus, mortality of *E. ironsidei* was 33% less with *C. griseum* and 22% less with *P. coccorum* than *B. bassiana*, which produced 100% mortality on *E. ironsidei* at 60 ppm conidial concentration.

The data indicate that *P. coccorum* and *C. griseum* are able to germinate upon and kill *E. ironsidei* in a laboratory environment. However, simple laboratory experiments cannot simulate the variable field environment (e.g., temperature, humidity, wind speed, and even soil moisture levels). The percentage of mortality caused by *P. coccorum* and *C. griseum* on *E. ironsidei* under field conditions remains to be quantified. However, results show promise that some level of field mortality

can be achieved and potentially may be high under suitable conditions.

Although *B. bassiana* GHA resulted in the best control of *E. ironsidei* in this study, local strains of this fungus have not been found naturally infecting *E. ironsidei* in Hawaii or elsewhere. *P. coccorum* and *C. griseum*, which were found naturally infecting *E. ironsidei* in macadamia orchards, may yet prove to be useful biocontrol agents for *E. ironsidei*. Inoculation of macadamia nut orchards with isolates of these fungi may result in the establishment of the fungi under the appropriate conditions. This approach has the potential to provide conservation biological control services.

Acknowledgments

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