MANIPULATION OF *HIRSUTELLA* AS A BIOLOGICAL CONTROL OF THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
A series of greenhouse experiments were conducted during 2005 to examine horizontal transmission of *Hirsutella homalodisca* infection to healthy GWSS. In addition, a series of different treatments were conducted to optimize the production of *Hirsutella*-infected GWSS. Hyphal body injection, topical spore application, and exposure to mycosed GWSS successfully produced both *Hirsutella* and *Beauveria* infections to a varying degree. Exposure to mummies collected this season was more efficient in disease transmission than exposure to last year's (frozen) cadavers. Results demonstrated that *H. homalodisca* is pathogenic to all GWSS stages and can be readily transmitted under glasshouse conditions. Furthermore, both our greenhouse and related field studies demonstrated that the fungus *Pseudogibellula formicarum* is a mycoparasite colonizing a high proportion of GWSS cadavers after mycosis by *H. homalodisca.*

INTRODUCTION
The goal of this research is to manipulate the primary GWSS mycopathogens as classical biological agents. Examples where this approach has been successful is the dissemination of the friendly fungus *Aschersonii* spp. for citrus whitefly control in Florida (McCoy et al.,1988) and the recent accidental introduction of *Entomophaga maimaiga* into gypsy moth populations (Hajek, 1999). In both cases, introductions of the mycopathogens resulted in successful long-term suppression of pest populations. This approach would be well suited for the introduction of highly fastidious pathogens and field and/or lab infected insects could serve as potential inocula. Over the past several years, field research has clearly demonstrated that the most common pathogen infecting in GWSS populations in southeastern US is the fungus *Hirsutella homalodisca* (Boucias et al., 2005). This subject area has great potential but has received virtually no research attention. Based on our preliminary experimentation and given the rapid colonization of CA by GWSS, the contributions of biological control (parasite spread) and the similarities of climate and weather (same horticultural zones occur in Florida and California) that are enabling the GWSS invasion of California, it is logical to conclude that the mycopathogens of GWSS collected from the Southeast will be equally adaptive to California.

OBJECTIVES
The major objective of this project is to identify the route of *Hirsutella homalodisca* disease transmission. A series of experiments have or will be conducted to determine the spatial and temporal factors required to transmit this agent from diseased to healthy insects under controlled greenhouse conditions. Specific experiments have examined:
1. Production of *Hirsutella* GWSS mummies.
2. Examining horizontal transmission of the disease. The transmission data will establish the protocols necessary to introduce this pathogen.
3. Analyzing the fate appearance and fate of *H. homalodisca* on the GWSS cadavers. The data on the overwintering biology of the pathogen in the GWSS mummies will provide insight into how this disease persists over a multi-seasonal time-frame.

RESULTS
During the field season of 2005 (June - September), varying numbers of adult GWSS (total = 490) from sweep-net collections in Quincy, Florida, were transported on sleeve-caged plants to the laboratory in Gainesville, Florida. Hemolymph samples were collected by removal of an antenna and examined for the presence of hyphal bodies. Healthy insects were maintained on potted plants placed in 1-m^3 screened cages in the greenhouse. Plants used for adult rearing were soy bean (*Glycine max* (L.) 'D90-9216'), cotton (*Gossypium hirsutum* L. 'Deltapine 88'), and cowpea (*Vigna unguiculata* (L.) 'California #5'). Leaves with egg masses were transferred to water agar Petri dishes and incubated at constant conditions (26 ± 1 °C, 12:12 h light/dark photoperiod, 85 ± 5% RH). Hatching neonates were transferred to caged lemon basil (*Ocimum basilicum* L. 'Lemon') plants in the greenhouse. Greenhouse temperature ranged from 26-30 °C, and indoor lighting maintained a 14:10 h light/dark photoperiod. The soil medium for all plants was watered to saturation once daily. For all experiments, individual plants were covered with clear acrylic cylinders (13 or 15 cm diameter x 45 cm high) with several holes (5 cm diameter) and the top covered with fine mesh gauze to allow air exchange. In order to introduce insects to the cylinders, the insects were chilled on ice for 15-30 min and transferred to filter paper, which was placed on the soil surface of

the plant pot. All bioassays were conducted in the greenhouse. In most experiments with nymphs, mortality was not recorded, since dead individuals could not be recovered from the soil. Mortality data for these assays refer only to mycosed cadavers found on the plant after termination of the experiment.

Second to fifth instar nymphs, reared on lemon basil from eggs, were injected with 50 nl of *H. homalodisca* hyphal body preparation (originating from in vitro cultures of strains 3A and 11B) or filter-sterilized Ringer's physiological solution for treatment or control, respectively. For nanoinjections, glass needles were mounted to a nanomanipulator. Adult GWSS were injected with 1 µl of hyphal body preparation (originating from different in vitro cultures of *H. homalodisca*) or filter-sterilized Ringer's physiological solution for treatment or control, respectively. Groups of 10-21 insects were transferred to a cylinder containing a host plant and maintained in the greenhouse for 3 weeks. Adult mortality was recorded daily and the hemolymph of dead individuals was examined for hyphal body propagation. After 3 weeks, all plants (from nymph and adult experiments) were examined for mycosed cadavers, and surviving GWSS were subjected to hemolymph examination. A total of seven injection experiments (three controls, four treatments) were conducted.

Different approaches were taken to apply spores of different fungi (*Hirsutella*, *Beauveria*, *Sporothrix/Pseudogibellula*) to healthy nymphs or adults of *H. coagulata*. Initially, spores of *Beauveria bassiana* (strain 6185) were suspended 0.005% Tween 80 and 1-µl droplets applied to the ventral surface of the thorax and abdomen of nymphs or adults. Control insects were treated with Tween 80 solution only. One group of adults was immersed in Tween 80 suspension for 5 sec. In a second series of assays, adult GWSS were treated by touching their ventral surface to sporulating colonies of different in vitro cultures of *Hirsutella*, *Beauveria*, *Pseudogibellula*, or *Verticillium*. A control group was exposed to UV-irradiated *Beauveria* spores. In a third series of bioassays, the ventral surface of adult GWSS was touched to sporulating GWSS cadavers displaying spores of *Hirsutella*, *Beauveria*, or *Sporothrix* (*Pseudogibellula*?). Groups of 5-20 insects were transferred to an acryl cylinder containing a host plant and maintained in the greenhouse for 3 weeks. Adult mortality was recorded daily, and dead individuals that had fallen onto the soil surface were removed to examine their hemolymph for hyphal body propagation. After 3 weeks, all plants (from nymph and adult experiments) were examined for mycosed cadavers, and surviving GWSS were subjected to hemolymph examination. A total of 15 different topical application experiments (four controls, eleven treatments) were conducted.

The majority of the experiments examined the ability to transmit fungal infection from field-collected, mycosed cadavers to healthy GWSS. During the first part of the season (until mid July), overwintered, weathered cadavers collected in January (stored at 4°C) were used. During the second part of the season, new cadavers collected in July and August were used. Cadavers were pinned to different plants (10-16 per plant), which were covered with an acrylic cylinder and maintained in the greenhouse. Groups of *H. coagulata* nymphs (N = 21-45) or adults (N = 12-16) were introduced to the cylinders and observed for mortality daily. Dead adults were removed from the soil surface and hemolymph samples were examined for the presence of fungal hyphal bodies. After 2-3 weeks, all plants (from nymph and adult experiments) were examined for mycosed cadavers, and surviving GWSS were subjected to hemolymph examination. Several plants with sporulating cadavers were re-used for additional exposure of healthy GWSS. A total of 20 cadaver exposure experiments were conducted. Groups of healthy adult GWSS subjected to antennal bleeding were used as controls for mortality comparisons.

Five out of 490 adults field-collected GWSS were diagnosed by antennal bleeds to be infected with *Hirsutella* hyphal bodies and all five died within four days post bleeding. The low incidence of disease in these samples corresponds to population/disease data collected from North Florida plots in 2005.

Hyphal body injection, topical spore application, and cadaver exposure treatments successfully produced *Hirsutella* and *Beauveria* infections in *H. coagulata*. However, infection rates varied greatly between different and among similar treatments. The injection of nymphs with 50 µl of a hyphal body preparation (glass needles) from strains 3A and 11B (collected 2003, 2004) did not result in infection. Of the 21 injected nymphs, 15 uninfected nymphs were recovered after 3 weeks. In the corresponding 19 control nymphs, 15 uninfected nymphs were recovered. Most likely, the dosage used was too low. The same hyphal body preparation caused 47% (7/15) infection when injected at 1 µl per inject into adult GWSS, and 93% of the injected adults died during the observation period of 3 weeks. However, no mycosis was observed. Mortality in the corresponding control was 67% (8/12). Injection of strain 6197 (collected 2005) resulted in 100% mortality and 93% (14/15) infection. Four infected adults mycosed on the plant. In the corresponding control assay, mortality was 70% (7/10), and the hemolymph one dead adult contained hyphal bodies (the same individual was scored negative 3 days before). Injection of a second preparation from strain 6197 resulted in 78% (27/30) mortality and 19% infection. Three of the five infected adults mycosed on the plant.

Using several different techniques, GWSS nymphs or adults were exposed to spores of different fungi (*Hirsutella*, *Beauveria*, *Sporothrix/Pseudogibellula*). Topical application of a *Beauveria* spore suspension produced low infection (8%, 1/12) in treated nymphs, whereas no infection was induced in adults (N = 20). Adult mortality did not differ between treatment and control (60 and 70%, respectively). Contact with sporulating colonies successfully transmitted *Beauveria* to adult GWSS producing 27% (12/45) infection, but no transmission was found with the poorly sporulating *H. homalodisca* cultures. Mortality in *Beauveria* treatments was significantly higher (91%, 41/45) than in *H. homalodisca* and control treatments (50

and 67%, respectively). Results from contact treatments with sporulating cadavers (*Hirsutella*, *Beauveria*, *Sporothrix/Pseudogibellula*) are pending. *Sporothrix*: no transmission, but 100% mortality within 11 d suggesting the production of toxins. The introduction of healthy GWSS to plants harboring either one-year old, weathered cadavers or this year's new cadavers resulted in transmission of *H. Homalodisca* infection within 3 weeks to both nymphs and adults of *H. coagulata*. The majority of the cadavers that were pinned to the plant displayed sporulating *H. homalodisca* mycelium within a week. This year's cadavers developed an unusually thick, white mycelium overgrowing the entire insect appearing like cotton balls. After 3-4 weeks, other fungi such as *Beauveria* and *Pseudogibellula* were observed on several cadavers or newly induced mycosed adults. Dead exposed nymphs or adults attached to the plant and displaying *Hirsutella*-induced mycosis were seen as early as 7 or 12 d after exposure in, respectively. Hyphal bodies were found in the hemolymph of dead adults as early as 8 d after exposure.

This year's cadavers were more efficient in disease transmission. When nymphs (regardless of instar) were exposed to this year's cadavers, no survivors were found and all dead insects were overgrown with *H. homalodisca* mycelium. The experiment was initially conducted with different instars on the same plant and repeated 4 times using an even-aged cohort of nymphs (neonate, 2nd, 4th, or 5th instar) each time. Exposure of nymphs to last year's cadavers yielded only 3 ± 6% infection (ranging from 0-10%). The mycelium growing on these mycosed nymphs was light and flat, not nearly as thick as on mycosed nymphs induced by this year's cadavers. Adult mortality after exposure to this year's cadavers (N = 4) was 96 ± 5% and significantly higher than in the corresponding controls (32 ± 37%); disease transmission was 48 ± 30% (ranging from 7-73%). Adult mortality in experiments using last year's cadavers (N = 4) was high (76 ± 8%) but disease transmission was 13 ± 11% (ranging from 0-25%), significantly lower compared with transmission from this year's cadavers.

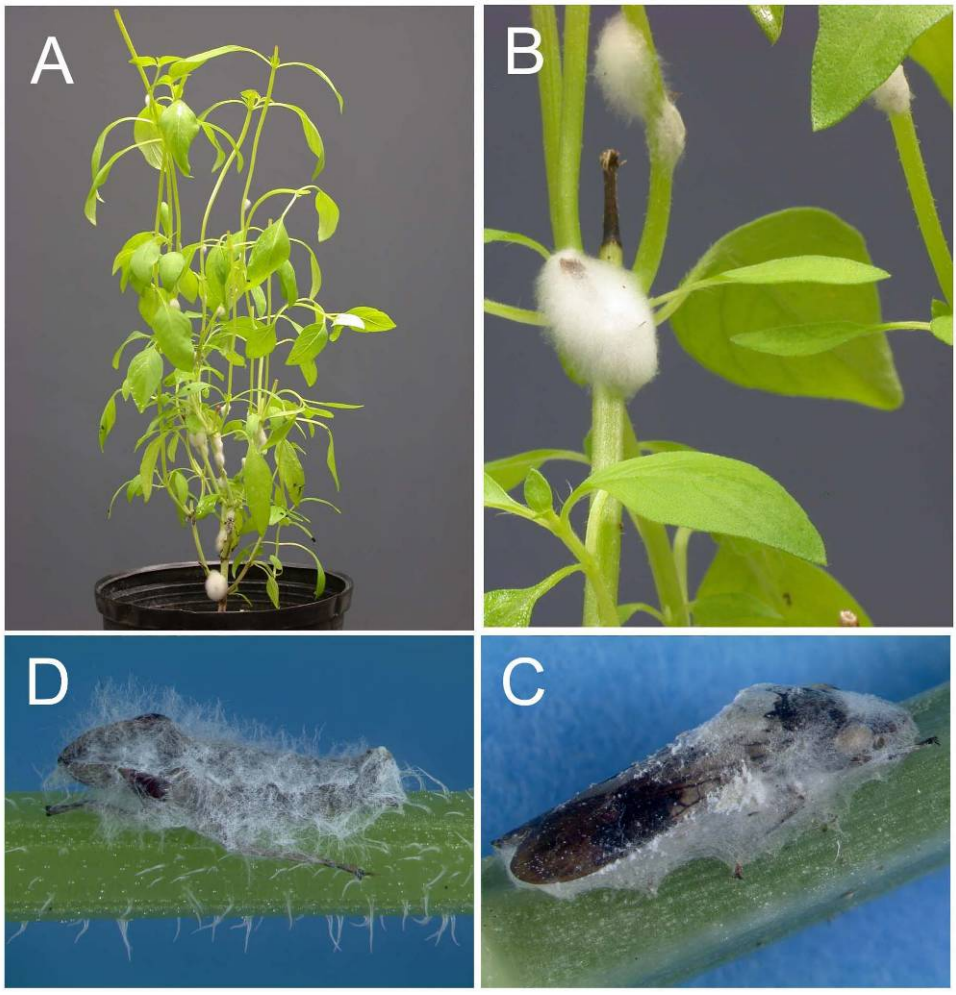

Figure 1. Cadaver exposure experiments. **(A)** Lemon basil plant 3 weeks after exposure of *H. coagulata* nymphs to this year's cadavers on a lemon basil plant. **(B)** Note the white, thick mycelium overgrowing the cadaver in the center and the introduced, mycosed nymphs. **(C)** Adult GWSS 4 weeks after exposure to last year's cadavers displaying *Hirsutella* mycelium and an emerging, secondary unknown (*Beauveria*?) mycelium. **(D)** Mycosed nymph 3 weeks after exposure of *H. coagulata* neonates to last year's cadavers on a lemon basil plant.

CONCLUSIONS

The research was directed at developing the technologies required to transmit *H. homalodisca* to healthy GWSS. Our findings demonstrated the following: 1) Kochs postulate was fulfilled; 2) the fastidious nature of *H. homalodisca* was confirmed; and 3) technologies required to amplify infectious material were established. Future research involving a combination of greenhouse and field studies will optimize *in vivo* produced *H. homalodisca* as an inoculum substrate.

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