Evaluation and Molecular Characterization of *Beauveria bassiana* for the Control of the Glassy-winged Sharpshooter (Homoptera: Cicadellidae) in California[1]

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Abstract The glassy-winged sharpshooter, *Homalodisca coagulata* (Say), is an important pest on grapes, citrus, almonds and other commercial crops in California as it is a vector of *Xylella fastidiosa* Wells, a bacterium that causes Pierce's disease in grapes, citrus variegated chlorosis, almond leaf scorch and other plant diseases. Various entomopathogenic fungi isolated from natural infections of *H. coagulata*, its habitats and other insect hosts were evaluated against this insect vector. Based on these studies, 3 isolates of the hyphomycetous fungus, *Beauveria bassiana* (Balsamo) Vuillemin, were selected for further evaluation. Two of these were California isolates, one each from the three-cornered alfalfa hopper, *Spissistilus festinus* (Say), and soil from *H. coagulata* habitat, and the third was a Texas isolate from natural infections of *H. coagulata*. All three were similar in their virulence to *H. coagulata* under laboratory conditions. The genetic relatedness of the *B. bassiana* isolates also was compared using single sequence repeat (SSR) markers which showed genetic diversity of this species based on the source of the isolate. Some isolates were 4× more infectious than others demonstrating that virulence of *B. bassiana* is not necessarily associated with their genetic relatedness.

Key Words entomopathogenic fungi, *Beauveria bassiana*, microbial control, microsatellite markers, sharpshooters, *Xylella fastidiosa*

The glassy-winged sharpshooter, *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), is an important agricultural pest in California vectoring the bacterium, *Xylella fastidiosa* Wells that causes Pierce's disease in grapes, citrus variegated chlorosis, almond leaf scorch and other diseases in various plant hosts that are of significant commercial value to California agriculture (Mircetich et al. 1976, Davis et al. 1978, Chang et al. 1993, Purcell 1995, Phillips 1998, Purcell and Saunders 1999). Although *X. fastidiosa* has been present in California vineyards for more than a century, the introduction of *H. coagulata* from the southeastern United States raised concerns over the spread of the disease threatening the multibillion dollar grape industry.

In response to the introduction of *H. coagulata*, a task force has been formed with federal and state agencies, universities, and trade and commodity organizations to

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develop strategies for managing the disease and vector problems. Biological control of *H. coagulata* is one tactic considered for long-term management of the pest and the bacterium it transmits (National Research Council 2004). Egg parasitoids (*Gonatocerus* spp.) have been released in California, and their impact is being evaluated (Pilkington et al. 2005). However, there is a need to exploit other natural enemies, like fungal pathogens, which invade the host by contact and are ideal for insect pests like *H. coagulata* which have piercing and sucking mouth parts. Information on the fungal pathogens of *H. coagulata* is limited, but researchers in the southeastern U.S. have conducted some preliminary studies evaluating the native fungal pathogens (Kanga et al. 2004, Mizell and Boucias 2005). The hyphomycetous fungi, *Beauveria bassiana* (Balsamo) Vuillemin, *Hirsutella homalodiscae* nom. prov. and *Pseudogibellula formicarum* (Mains) Samson & Evans, were recovered from *H. coagulata* in Texas, Mississippi, and Florida. We evaluated these pathogens in the preliminary studies (Dara et al. 2004, 2005 and 2007) and, based on the virulence and ability to grow at high temperatures, selected 3 isolates of *B. bassiana* for further evaluation. We report herein the virulence of these isolates against *H. coagulata*. We also determined the genetic relatedness of some isolates of *B. bassiana* using single sequence repeat (SSR) or microsatellite markers.

Materials and Methods

Assays with *B. bassiana* isolates. Three isolates of *B. bassiana*—two California isolates one each from the three-cornered alfalfa hopper, *Spsissistlus festinus* (Say), and the soil from a citrus orchard in Riverside, and the Texas isolate from *H. coagulata* from Weslaco—were evaluated at 3 concentrations (10^9, 10^7 and 10^5 conidia/ml) against laboratory-reared adult *H. coagulata* obtained from California Department of Food and Agriculture, Riverside, CA. Fungal isolates were cultured on Sabouraud dextrose agar medium enriched with 0.2% yeast extract at 25°C. Conidia were harvested by scraping the sporulating cultures with a sterile spatula and transferred to sterile distilled water containing 0.01% Silwet L-77 (Loveland Industries, Inc., Greeley, CO), an adjuvant. Suspensions were filtered through sterile cheesecloth, and conidial concentrations were determined using a hemacytometer. Viability of the conidia was verified prior to each experiment by the proportion germinated after 16 h of incubation in potato dextrose broth at room temperature on a rotary shaker. Final concentration of the suspension was adjusted based on conidial viability.

To initiate the treatments, insects were anesthetized by exposing them to CO_2 for 20 sec. Individual insects were inoculated by rolling each insect in a 10 µL droplet of conidial suspension. Untreated insects and those treated with 0.01% Silwet L-77 solution were used as controls. Each treatment had 20 adult insects incubated on potted cowpea plants in cylindrical acrylic cages (35 cm height × 17 cm diam) with screen tops at 27°C and 16L:8D photoperiod. Mortality of insects was monitored daily for 2 wks. Each insect cadaver was surface sterilized in 3% sodium hypochlorite solution (followed by rinsing in deionized water) and incubated on 1% water agar for fungal emergence. Identity of the fungus was confirmed by microscopic examination following a key (Humber 1997), and those cadavers with *B. bassiana* emergence were considered infected. This experiment was repeated twice at monthly intervals.

Another set of assays was conducted using the 3 selected *B. bassiana* isolates where field-collected adult *H. coagulata* were released into cages with treated cowpea plants. Four-week-old cowpea plants were sprayed with 1×10^{10} viable conidia/

plant in 40 ml of 0.01% Silwet L-77 to the point of run off and allowed to dry for 15-20 min before placing them individually in screened cages (BugDorms by BioQuip Products, Inc., Rancho Dominquez, CA). Each isolate had one such cage with a single plant; 50 adult *H. coagulata* were released into each cage without anesthetization. A plant treated with 0.01% Silwet L-77 was used as a control. Cages were maintained under laboratory conditions where temperature was 26.1 ± 4.0°C and relative humidity fluctuated between 36 and 62% with an average of 42%. Insect mortality was monitored daily for 2 wks following the procedures previously described. This experiment was repeated 3× at 2-3 week intervals.

Data from the assays with *B. bassiana* isolates were analyzed using ANOVA, and significant means were separated by Tukey's studentized range (HSD) test at $P < 0.05$ (Statistix 8 2003).

Molecular characterization. SSR markers developed by Rehner and Buckley (2003) were used to determine the genetic relatedness of 7 *B. bassiana* isolates in our study. They included, in addition to the 3 isolates used in the virulence study, one each from the California harvester ant, *Pogonomyrmex californicus* (Buckley) Kern Co, soil from a citrus orchard in Tulare Co, CA, *H. coagulata* in Jackson, MS, and a commercial isolate GHA (Laverlam International Corp., Butte, MT). Fungal cultures were grown on Sabouraud dextrose agar enriched with yeast extract, and DNA was extracted using MagAttract 96 DNA Plant kit (Qiagen, Valencia, CA) and a Rectsch MM301 Mixer Mill (Retsch, Germany) following the protocols described by McGuire et al. (2006). Seven PCR primer pairs (Ba01, Ba02, Ba03, Ba05, Ba06, Ba08, and Ba12) which flank SSR markers were used for the molecular characterization of these fungal pathogens.

To evaluate the pattern of genetic similarities among the selected isolates of the fungal pathogens, pairwise genetic similarity coefficient was calculated based on Jaccard's similarity coefficient (Jaccard 1908). A dendrogram was constructed using the neighboring join (N-J) clustering analysis (Saitou and Nei 1987) with midpoint rooting method. All statistical analysis and the construction of the dendrogram were performed using the numerical taxonomy and multivariate analysis system (NTSYS-pc) version 2.1 (Rohlf 2002).

Results and Discussion

Previous studies reported 2 California isolates of *B. bassiana* – one each from *S. festinus* and a soil sample from a Riverside citrus orchard, and the Texas isolate were found to be more virulent than the other isolates tested (Dara et al. 2007). Virulence of these 3 isolates was similar within each concentration tested (Table 1) or in the caged studies (Table 2). Cage studies demonstrate the potential of *B. bassiana* to infect *H. coagulata* feeding on treated plants. Although the difference was not significant ($P > 0.05$), the Texas isolate seemed to cause low infection in cage studies. Since emergence of the pathogen from the infected host is important for the dispersal and survival of the pathogen in host populations, CA isolates might be more suitable for microbial control of *H. coagulata*. Citrus is a favorable host and serves as a source of overwintering populations of *H. coagulata* that spread to other hosts in the spring (Perring et al. 2001, Castle et al. 2005). Introducing *B. bassiana* into overwintering *H. coagulata* populations can be an ideal strategy in two ways. First, cool winter months are suitable for fungal infections and, secondly, *B. bassiana* is a soilborne fungus and can survive in the habitat to serve as a source of infection for overwintering adults that

Table 1. Virulence of the selected *Beauveria bassiana* isolates to *Homalodisca coagulata* at different concentrations

Isolate	Concentration (conidia/ml)		
	10^9	10^7	10^5
	Percent mortality (corrected)*		Mean ± SD**
CA-Hopper	75.6 ± 7.9	51.2 ± 5.3	30.4 ± 14.6
CA-Soil 41	57.6 ± 31.9	53.8 ± 38.8	36.9 ± 9.7
TX-Sharpshooter	71.5 ± 22.5	29.4 ± 20.3	23.1 ± 11.4
	F = 1.21	F = 0.73	F = 15.04
	Percent infection		Mean ± SD**
CA-Hopper	57.5 ± 17.6	38.2 ± 16.7	7.9 ± 11.1
CA-Soil 41	36.0 ± 8.5	10.2 ± 0.3	0 ± 0
TX-Sharpshooter	47.5 ± 3.5	20.0 ± 14.1	2.5 ± 3.5
	F = 2.03	F = 1.68	F = 0.55

* Percent mortality was corrected based on the control mortality (5.1 ± 0.1).
** Means within a column are not significantly different (df = 2, 5, $P > 0.05$).

fall to the ground and next generation nymphs that crawl on the ground as they spread to other trees.

Molecular characterization showed that the Riverside soil isolate (41) of *B. bassiana* was more closely related to the commercial isolate than to the other California isolates (Fig. 1). Whereas California isolates from insect hosts were closely related, the Tulare soil isolate (25) was distinctly separated from all isolates used in our study. Texas and Mississippi isolates also were separated from the rest, showing genetic diversity of this species based on the source of the isolate. Although the differences were not significant, the isolates from the Riverside soil and the three-cornered alfalfa hopper were 3× to 4× more virulent than the commercial, the ant and the Tulare soil

Table 2. Virulence of the selected *Beauveria bassiana* isolates to *Homalodisca coagulata* in caged assays at 1 × 10^{10} conidia/plant

Isolate	Mean ± SEM*	
	Percent mortality**	Percent infection
CA-Hopper	84.1 ± 12.4	56.7 ± 17.4
CA-Soil 41	71.7 ± 3.0	48.6 ± 9.0
TX-Sharpshooter	59.1 ± 18.1	10.5 ± 0.8
	F = 1.54	F = 4.57

* Means within a column are not significantly different (df = 2, 8, $P > 0.05$).
** Percent mortality was corrected based on the control mortality (24.8 ± 1.9).

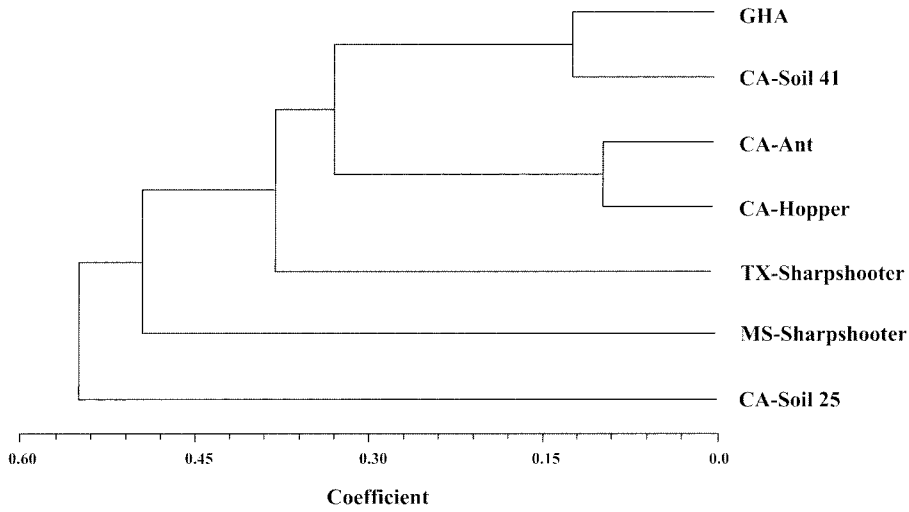

Fig. 1. Genetic relatedness of the isolates of *Beauveria bassiana* based on seven SSR markers. Neighboring join (N-J) clustering analysis with midpoint rooting method was used to construct the dendrogram.

isolates (Dara et al. 2007) demonstrating that virulence of the pathogens is not necessarily associated with genetic relatedness. Molecular characterization of the fungal isolates provided a better understanding of their relatedness.

These assays demonstrate the potential of *B. bassiana* isolates and we propose further testing of the 3 isolates for the microbial control of *H. coagulata* in California.

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