Managing A Challenging Subterranean Clover Pest: Sustainable Control Using Insect Pathogens

Final Report for GW15-018

Project Type: Graduate Student
Funds awarded in 2015: $12,859.00
Projected End Date: 12/31/2015
Grant Recipient: Oregon State University
Region: Western
State: Oregon
Graduate Student:

Anis Lestari

Major Professor:

Dr. Sujaya Rao
Oregon State University

Project Information

Summary:

Insect pathogens provide an effective means of suppressing pests but have received less attention compared with other biological control agents. For pests that develop below ground, insect pathogens may offer the best management option. My goal was to investigate the virulence of insect pathogens against the clover root borer, an economically important and unique bark beetle pest inadvertently introduced into the United States over 100 years ago. While bark beetles typically develop in the trunks of trees in forests, the clover root borer develops belowground within the roots of red clover. In my study, red clover seed fields were surveyed for the presence of naturally occurring entomopathogens and their virulence was evaluated against clover root borers. Based on morphology of spore bearing stuctures and sequences of ITS regions of fungal isolate genes, *Beauveria bassiana, B. pseudobassiana, Isaria fumosorosea, Lecanicillium muscarium*, *Metarhizium anisopliae* var *anisopliae, M. robertsii, M. quizhouense* and *M. brunneum* were detected in red clover seed fields. This is the first report of these entomopathogenic fungi in red clover fields in western Oregon. A laboratory study conducted with *B. bassiana* (isolate FD) documented that at high spore concentrations fungal spores are transmitted horizontally which facilitate spread of the disease pathogen. A second laboratory bioassay documented that field-isolated *Beauveria bassiana* and *Isaria fumosorosea* had similar levels of virulence compared to commercial products, *Metarhizium anisopliae* and *Isaria fumosorosea* that were tested. For determining the impact when clover root borers are exposed to entomopathogens in field collected soil, *Beauveria bassiana* (isolate FD) and *Metarhizium anisopliae* var *anisopliae* (isolate A4-MA) were evaluated. The study showed that *Metarhizium anisopliae* successfully established a colony, sporulated in the soil, and infected

clover root borer adults while growth, development, and infection by *Beauveria bassiana* was minimal. Based on these studies, entomopathogenic fungi have potential for use as a biological control agent for clover root borer pests in red clover seed fields in western oregon.

Introduction

The clover root borer, native to Europe, was accidently introduced into the U.S. over 100 years ago. Early studies were focused on gathering information related to its biology, development, and damage to red clover (Westgate and Hillman 1911; Rockwood, 1926). Subsequent studies evaluated control strategies using organochlorine insecticides which killed the pest (Gyrisco and Marshall 1950; Cyrisco et al. 1954; Preuss and Weaver 1958; Koehler et al. 1961). These insecticides had long residual action and were thus effective in killing the pest during the short period when adults emerged from the roots below ground and dispersed to infest new hosts. After the ban on use of organochlorines, the newer insecticides that were developed had shorter residual action for mitigating negative impacts on the environment, but none were effective for controlling the pest. Since the ban on use of this group of insecticides, the pest has received little attention due to the challenges of developing a management strategy given its subterranean development. Insecticides currently registered for red clover are not effective against the pest (Rao et al. 2012). In Chile, new cultivars are being evaluated against the pest (Alarcón et al. 2010), but in the U.S., no efforts are being directed towards examination of host plant resistance. Meanwhile, entomopathogenic fungi and nematodes have been found to be effective against diverse beetle pests when applied to the soil or when incorporated into an autoinoculation strategy (Vega et al. 1995; Lacey and Shapiro-Ilan 2008).

Project Objectives:

1. Determine the presence and abundance of naturally occurring soil dwelling insect pathogens of the clover root borer.
2. Compare the virulence of entomopathogens of the clover root borer:

- Assess virulence of entomopathogenic fungi against the clover root borer
- Assess virulence of entomopathogenic nematodes against the clover root borer
- Assess horizontal transfer of entomopathogens in the clover root borer

Cooperators

- Anis Lestari

  anislestari1@gmail.com

  Graduate student

  Oregon State University

  3017 ALS Soil Sciences
  Oregon State University

Corvallis, OR 97331

Research

Materials and methods:

The following studies were conducted with entomopathogenic fungi. Studies conducted with entomopathogenic nematodes could not be completed due to lack of viability of nematode cultures.

Study System. The study was conducted in western Oregon where red clover seeds are produced for use nationwide.

Survey and isolation of insect pathogens. Over two years, soil samples were collected from five red clover seed production fields (Table 1) in the Willamette Valley. Clover root borers were collected from the soil samples and pathogens observed growing on dead adults were isolated and plated on appropriate media. In addition, soil baiting (Zimmerman, 1986) was used for isolating pathogens from soil samples. Soil baiting was accomplished by exposing surrogate insect hosts, larvae of wax moths (*Galleria* sp.), to rhizosphere soil samples collected from the fields.

In a preliminary survey conducted in year 1, five dead adult clover root borers extracted from red clover roots from Field 1 were observed to be infected with fungal entomopathogens. For confirmation of the presence of the entomopathogenic fungi in red clover fields, 20 surrogate *Galleria* larvae were exposed to four rhizosphere soil samples collected from the same field. In year 2, 25 roots with rhizosphere soil were collected and bagged from three random locations in each of four fields using a 4m x 4m grid. From each bag, 10 soil samples from 10 - 13 roots were randomly selected and transferred to sterile petriplates (85 x 15 mm) to which five surrogate *Galleria* larvae were added. After two weeks of exposure, dead *Galleria* larvae were rinsed with distilled water and then placed on wet filter paper to allow for development of fungal entomopathogens. Fungi that emerged from each cadaver were isolated by inoculating the spores and/or mycelia on Potato dextrose Agar (PDA, Difco) containing antibiotic chloramphenicol (0.2 % in 1 liter media). Isolated fungal cultures were then examined under the microscope for identification to the genus level based on the morphology of spore bearing structures (conidiophores).

Molecular identification. Fungal identities were confirmed using molecular techniques. Twelve entomopathogenic fungus were selected for DNA extraction. Spores and/or hyphae were grown on Sabouraud Dextrose Agar (SDA, Difco) media layered on top with a piece of sterile Whatman filter paper (diametre 42.5 mm) in a petriplate (55 x 1.5 mm) using the protocol described by Kepler et al. (2014) with slight modifications. The cultures were maintain at 28°C for 5-7 days. Each isolate was then harvested by transferring to a sterile 1 ml microcentrifuge tube. The tissue was then frozen in liquid nitrogen, and ruptured by using blue mecro pestles. About 20-50 mg of fungal tissue was used for DNA extraction with DNAeasy Plant Mini kits (50 preps). Extracted DNA samples were frozen at -20^{0}C until used.

GoTaq green mastermix (Promega) was used to amplify the Internal Transcribed Spacer region (ITS) of rRNA gene. In the PCR amplifications, ITS-1F (5'-CTTGGTCATTTAGAGGAAG TAA- 3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') were

used as the forward primer and reverse primer, respectively (White et al. 1990; Gardes and Burns 1993). PCR reaction was initiated with denaturation at 95^0C for 2 minutes followed by thirty five cycles of 94^0C for 40 seconds, annealing at 52^0C for 1 minute, 72^0C for 1 minute thirty seconds, and final extension at 72^0C for 7 minutes.

PCR products were purified by using Qiagen QIAquick PCR purification kit and sent to the Genome Research and Biocomputing (CGRB), Oregon State University for sequencing. Similar primers mentioned above were used for sequencing the ITS region. The fungal sequences attained were used for performing BLAST searches to match and confirm their identification with submitted reference strains in NCBI GenBank.

Preliminary virulence test of field isolated fungi. Six fungi including two strains of *Beauveria bassiana* (isolate FD and W1) and two strains of *Isaria fumosorosea* (isolate W4 and W6) isolated from red clover fields, and two commercial fungi, *Metarhizium anisopliae* (F52) and *I. fumosorosea* (FE 9901), obtained from the USDA-ARS lab in Corvallis, OR, were inoculated on Sabouraud Dextrose Agar (SDA) media. After 10-14 days, spores of each isolate were harvested by flooding with 12 ml of a sterile 0.1% Tween 80 solution followed by gentle scraping of the spores with a sterile inoculation needle for separation from fungal media surfaces. Each harvested spore isolate was sieved by passing through a double layer of cloth to separate the spores from the mycelia. The harvested spores were then counted using a hemocytometer and the concentration of the solution was adjusted to 10^8 spores/ml by addition of new harvested spore suspensions if needed.

The laboratory bioassay was conducted using the dip method. To each spore suspension, ten adults of clover root borer were immersed for 5 - 7 seconds. The adults were air dried and then placed on white sterile sand in petri plates (15 x 45 mm). A solution of Tween (0.1%) was used as a negative control. The plates were placed in an incubator in dark at a constant temperature of 22°C and humidity 60-70%. The experiment set up as a randomized block design with six replicates. The dishes were monitored daily and the number of dead adults was recorded for two weeks. Each dead adult was placed on wet filter paper to observe fungal growth, if any, on the cadaver.

Horizontal transfer study. *Beauveria bassiana* (isolate FD) isolated from died field collected clover root borers was inoculated in PDA media and maintain for 10-14 weeks at room temperature. The spores were harvested by pipetting 10 ml of a sterile of 0.1% Tween solution onto a culture plate and gently separating spores from agar media using an inoculation loop needle. The spores were counted with a hemocytometer and low (1×10^5 spores/ml) and high (9.7×10^7 spores/ml) concentration solutions were prepared.

One clover root borer adult was dipped in the low spore concentration solution for about 5-7 seconds and then air dried on paper towels. The infected borer was then exposed to nine undipped borers in a small petridish (15 x 45 mm) layered on the bottom with white filter paper. Similar infection procedure was conducted for exposure to the high concentration level. The experiment was conducted as a randomized block design with five replications. Adult mortality was recorded after 21 days.

Laboratory bioassay with field collected soil. *Beauveria bassiana* (isolate FD) and *Metarhizium anisopliae* var *anisopliae* (isolate A4-MA) were inoculated on SDA media and incubated for two weeks at room temperature. The spores of each isolate were

harvested as described above. The spores of each isolate were counted with a hemocytometer to determine their initial concentration. Subsequently, the concentration was adjusted to four concentrations (10^4, 10^5, 10^6, 10^7 spores/ml). A sterile Tween 80 solution (0.1%) was used as a negative control.

Spore solutions of each isolate at each of the four concentrations (10^4, 10^5, 10^6, 10^7 spores/ml) were added to separate ziplock bags filled with 175 gram field soil to reach 27% (w/w) of soil moisture. The soil and spore suspension inside the ziplock bag was mixed by hand and then transferred to small cups (40 x 27 x 35 mm)which were then closed with lids. To each cup, 35 grams of inoculated soil was added. Five clover root borer adults rinsed with distilled water were then transferred to the inoculated soil. The cups were placed in an incubator with no light at a constant temperature of 22^0C, and 60-70% humidity. The experiment was conducted as a randomized block design with five replications. As a negative control, the soil was inoculated with a sterile 0.1% of Tween 80 solution. The number of dead adults was recorded after two weeks.

Data analysis. The data was subjected to Pearson chi-square test to compare the impacts of the fungal treatments on clover root borer adults. To determine differences across treatments, the adjusted residuals were calculated with Bonferroni adjustment (Sharpe, 2015). Data analysis was performed using IBM SPSS 22.

Research results and discussion:

Survey and isolation of insect pathogens. Naturally occurring entomopathogenic fungi isolated from five red clover fields in the Willamette Valley, Oregon, belonged to four genera, namely *Beauveria, Metarhizium, Isaria* and *Lecanicillium* based on the morphology of spore bearing structures (Table 1). In all, more than 150 isolates of entomopathogenic fungi were found infecting surrogate *Galleria* larvae exposed to rhizosphere soil. The most common fungi belonged to the genus *Beauveria* (more than 100 isolates collected across all five sites), followed by *Metarhizium* (29 isolates from three sites). *Isaria* and *Lecanicillium* were found only at two and one site, respectively. This is the first study to document the presence of four genera of entomopathogenic fungi in western Oregon fields.

Molecular identification. Based on sequences of ITS 1F and ITS 4, DNA barcoding using Blast search, eight naturally occurring entomopathogenic species were identified from twelve field isolates collected from the five red clover seed fields. These included *Beauveria bassiana, B. pseudobassiana, Isaria fumosorosea, Lecanicillium muscarium*, *Metarhizium anisopliae* var *anisopliae, M. robertsii, M. quizhouense* and *M. brunneum* (Table 2).

Preliminary virulence test of field isolated fungi. Both the commercial products and field isolates infected more than 50% of the clover root borers when exposed to each treatment in petri dishes. After six days post-infection, the commercial fungus *Metarhizium anisopliae* killed more than 80% of treated adults (Figure 1). Also, all field isolated fungal strains of *Beauveria bassiana* (isolate FD and W1) as well as the commercial *Metarhizium anisopliae* caused 100% mortality.

Horizontal transfer of *B.bassiana.* A field isolated entomopathogenic fungus, *Beauveria bassiana* (isolate FD) was tested for its ability for disseminating its spore from one CRB in to another one. After 21 days of exposure, high level of spore concentration infected more adults than the low spore concentration treatment and

the control. The high spore concentration treatment infected 34 clover root borers, whereas only 12 adults died when they were treated with low level of concentration (Table 3). Additionally, there was no difference in mortality of dead adults in the low concentration level compared to the control. It is possible that the control beetles were contaminated with the pathogen.

Laboratory bioassay with field collected soil. The effect of spore concentration of *Metarhizium anisopliae* on clover root borer mortality in soil is presented in Figure 2. Pearson Chi-Square test revealed that there was a significant different in clover root borer mortality across the *Metarhizium* concentrations tested *Metarhizium* (Pearson $X^2 = 94.5$, 8 df, p-value < 0.05). Of the four concentrations, the highest concentration casued significantly higehr clover root boer mortality compared to the other concentrations (Pearson $X^2 = 73.6$, 1 df, $p < 0.003$).

Metarhizium anisopliae successfully established a colony, well-sporulated in the soil, and infected clover root borers at the highest spore concentration used for inoculating field soil. However, high numbers of clover root borer cadavers with white spores (showed mycosis symptom caused by *Beauveria*) were also recovered in this trial. Based on the survey described above, *Beauveria* was present in all red clover fields surveyed and is likley to have contaminated the *Metarhizium* experiment as the soil used in the trial was not autoclaved.

In a similar study conducted with *Metarhizium anisopliae* isolate F52, more than 80% mortality of *Delia radicum* larvae was observed when soil inoculation concentration was 3.85×10^6 spores/ml (Bruck et al, 2005). The difference could be due to difference in pest response to the fungus or difference in the stage of the pest exposed to the fungus.

In the trial with *Beauveria bassiana,* statistical analysis showed that, at the highest level of concentration, clover root borer adult mortality was not significantly different with other concentrations and the control (Pearson $X^2 = 0.52$, 1df, $p > 0.003$) (Figure 3). This indicates that *Beauveria bassiana* inoculation is not as effective as *Metarhizium anisopliae* against clover root borer under the conditions of the experiment. The basis for the difference is not known as in the previous laboratory experiment described above, *Beauveria bassiana* was as effective as *Metarhizium anisopliae.*

- Figures and Tables

Participation Summary

Educational & Outreach Activities

PARTICIPATION SUMMARY:

Education/outreach description:

Lestari, A.S., and Rao, S. 2015. Entomopathogenic fungi as a potential biocontrol strategy for clover root borer management. Research report. 74th Annual Pacific Northwest Insect Management Conference. p.39

Lestari, A.S., and Rao, S. 2014. Potential for management of the clover root borer pest in red clover seed production fields using insect pathogenic fungi. In Nicole, A., Ed., Seed Production Research, OSU: 27-29

Presentations

This research was presented at the following meetings:

- Entomological Society of America meeting, Portland, OR, November 2014.
- Pacific Northwest Insect Management Conference, Portland, OR, January 2015
- Annual Student Research in Entomology Graduate Symposium, Corvallis, OR, April 2015
- Pacific Branch meeting of the Entomological Society of America, Couer d'Alene, ID, April 2015.
- Hyslop Farm Field Day, Corvallis, OR, May 2015

Project Outcomes

Project outcomes:

This is the first study to document the presence of four genera of entomopathogenic fungi in western Oregon fields. A single report (Rockwood 1926) exists of an entomopathogen, *Beauveria globulifera*, associated with the clover root borer. In studies by Bruck (2010) and Fisher et al. (2011) only *Beauveria* and *Metarhizium* were detected. We speculate that more entomopathogenic fungi were detected in the current study due to exposure of the rhizosphere soil to surrogate *Galleria* larvae instead of root samples as *Isaria* and *Lecanicillium* are both soil-inhabiting fungi.

This is also the first study to examine the virulence of entomopathogenic fungi against the clover root borer. *Beauveria bassiana* has been tested against another red clover pest, *Sitona lepidus* in which it caused up to 100% mortality after 2-4 weeks post infection (Willoughby et al. 1998).

Based on the current study, *Metarhizium* has the greatest potential to use as a biological control agent against the clover root borer. It was the second most dominant naturally-occurring fungus recorded from red clover fields, killed 100% of clover root borers in the virulence test, and successfully established a colony, sporulated in the soil, and infected more than 80% clover root borer adults in the bioassay with field collected soil.

Economic Analysis

A field study is needed for an economic analysis of the use of entomopathogens for management of the clover root borer in red clover seed production fields. The cost of the product and its application will need to be compared with the cost of removing the crops when damage is high as currently there are no management options for this pest. A field trial was beyond the scope of the current study.

Farmer Adoption

The study documented that the field isolated fungus, *Metarhizium anisopliae*, has potential for use as a biological control agent against the clover root borer pest. A large scale field test needs to be conducted before a recommendation can be made for farmer adoption.

Recommendations:

Areas needing additional study

Further research is needed for development of entomopathogenic fungi as biological control agents for the clover root borer. A range of doses need to be evaluated in commercial red clover seed production fields, followed by an economic analysis of the cost of applying the dose with the greatest potential. The field tests should be done with both *Metarhizium anisopliae,* which documented the greatest potential, and with *Beauveria bassiana* which was the most dominant fungus isolated from red clover seed fields in western Oregon.

- References