New York Greengrass Association / Turfgrass Environmental Stewardship Fund

Progress and Final Report for **1/1/2017 - 3/31/2017**

Project Title: **Biological Control of Annual Bluegrass Weevil with novel Formulation Types and Application Systems for Entomopathogenic Fungi: Microsclerotia-based formulations and Hydrogels**

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Summary: Under laboratory conditions we tested the effect on the entomopathogenic fungus *Metarhizium brunneum* F52 of different concentrations of 5 commonly used fungicides incorporated into water agar in Petri dishes. Chlorothalonil did not inhibit the growth of the fungus; iprodione showed slight inhibitory effect at higher concentration; propiconazole, TwinLine and Stratego strongly inhibit the fungal growth except at the lowest concentration of 1 mg/L. Under greenhouse conditions, chlorothalonil, iprodione, and propiconazole were sprayed on pots with creeping bentgrass that had been inoculated with *M/ brunneum* F52. Only propiconazole at the high rate had a slightly suppressive effect. Under field conditions, any suppressive effect on the *M. brunneum* a likely to be reduced. Spores produced applied from microsclerotia granules of *M. brunneum* F52 caused high mortality of ABW adults in Petri dishes in the lab. But in the greenhouse in pots with creeping bentgrass, the fungus had no significant effect. In pots with *P. annua* in the greenhouse, the fungus had only a limited effect on ABW larval populations. However, because entomopathogenic fungi have been synergized against several other insect pest with imidacloprid, we will explore such combination with and without hydrogels in field tests in spring.

OBJECTIVES

The long term goal is to develop a granular formulation of microsclerotia of *Metarhizium anisopliae* F52 as an effective and viable biological control option for ABW. The here proposed research is to lay the foundations for this goal by addressing these objectives:

1. Determine compatibility of formulation with commonly used golf course fungicides.
2. Determine efficacy of formulation against ABW adults and externally feeding larvae.
3. Disseminate generated knowledge to the golf course industry.

Background. The annual bluegrass weevil (ABW), *Listronotus maculicollis*, is the most important and difficult to control insect pest of short-mown golf course turf and grass tennis courts in eastern North America. In a recently conducted survey throughout the area affected by the pest, for New York State 29% of participants suspected resistance with regionally up 55%

(Long Island) (McGraw & Koppenhöfer 2017). Against highly resistant ABW populations, no adulticides and as few as two larvicides are still effective (Koppenhöfer et al. 2012; Koppenhöfer, unpubl. data).

Entomopathogenic fungi. Entomopathogenic fungi are used successfully for a number of insect pests, but they had so far only shown limited potential for ABW control. A product based on the fungus *Beauveria bassiana* GHA strain (BotaniGard) has been ineffective when applied against externally feeding larvae and provided variable and limited control when applied against overwintered adults (0-40%) (AMK, unpubl. data). The fungus *Metarhizium brunneum* F52 strain (formerly *M. anisopliae*) has provided 0-46% control applied against externally feeding larvae (Ramoutar et al. 2010). All the above fungal formulations were based on the conidial spores, the infective propagules of these fungi.

Promising new delivery methods for entomopathogenic fungi. Recent advances in the *in vitro* production of entomopathogenic fungi as a microsclerotium, rather than conidial spores, should result in more cost competitive products. Microsclerotia are naturally formed in soil by fungi as survival structures consisting of melanized hyphae. *In vitro* produced microsclerotia can be dried and formulated into granules. When these granules hydrate, the microsclerotia produce infective conidial spores over several weeks, significantly increasing the residual activity compared to conidial spore applications. Experimental samples of *M. brunneum* F52 strain microsclerotia have already shown potential for the control of black-legged tick, sugar beet root maggot, and Japanese beetle (Behle et al. 2013, 2015). The soil/thatch surface below the turf canopy should offer an excellent environment for microsclerotia granules to produce infective conidial spores and do so over an extended period of time.

Further, improvements in conidia production by microsclerotia in soils can be made by the addition of hydrogels. Hydrogels is the collective term for synthetic polymers (acrylate or cellulose-based) in the form of crystals or tiny beads that are available under several trade names such as water-holding crystals, super absorbent polymers, flower crystals (Ahmed 2015). Hydrogels have a capacity to hold large volumes of water when moistened and can slowly release this retained water over time, making it available to plants, fungi and other organisms (Demitri et al. 2013).

Effect of fungicides on entomopathogenic fungi in turfgrass. Fungicide are widely used on the short mown areas of golf courses, particularly on greens and collars, and to lesser extent on fairways and approaches. To develop these fungi as biocontrol agents for ABW, it is crucial to determine how compatible they are with the commonly used fungicides on golf courses.

General methodology. ABW adults were collected from overwintering sites in October/November at 2 sites with susceptible ABW (Rutgers Hort. Farm 2, North Brunswick NJ; Pine Brook GC, Manalapan, NJ) and 1 golf courses with confirmed high level of pyrethroid resistance (Preakness Hills CC, Wayne, NJ). Adults were kept on pasteurized moist sand for 2–6 months at 10 h light at 6°C : 14 h dark at 4ºC. Prior to bioassays, adults were extracted and kept in on moist sand at 14 hr light at 21°C: 10 h dark at 14°C for at least 10 days with food.

A clay-based granular formulation of *M. brunneum* F52 microsclerotia (1.45×109 colony forming units/g) was provided by Dr. Mark Jackson at the USDA-Agricultural Research Service, Crop Bioprotection Research Unit in Peoria, IL.

For experiments with adults, we used creeping bentgrass (cv. L-93) grown in the greenhouse for 2 months prior to use. *Poa annua* for larval experiments was grown from plugs taken from uniform established fields with no history of ABW infestations. *Poa annua* plugs were pressure

washed free of soil and grown in the greenhouse for 4 weeks before experiments. Plants were fertilized weekly, watered as necessary and clipped 2x/wk (0.5" height).

To obtain turf infested with ABW larvae for the efficacy testing, *P. annua* established from plugs was fitted into cups with screen-covered ventilation holes and 4 male and 4 female ABW caged in the cups for 1 week for egg-laying. After adult removal, pots were be left in the greenhouse for larvae to develop.

Obj. 1. Compatibility of formulation with commonly used golf course fungicides.

Laboratory tests

Methods. The compatibility of *Metarhizium brunneum* F52 microsclerotia with five fungicides was tested under laboratory conditions. Representative commercial fungicide formulations from the major fungicide classes were incorporated into water agar at rates that included and exceeded the typically used field rates of these fungicides (1, 10, 100, 250, 500, 1000 mg/L agar). The fungicides were Banner Maxx II (AI: 14.3% propiconazole), Chipco 26 GT (iprodione), Daconil WeatherStick (54% chlorothalonil), TwinLine (12% pyraclostrobin and 7.4% metconazole), and Stratego (11.4% propiconazole and 11.4% trifloxystrobin). There were 5 Petri dishes per fungicide-rate combination in each of three experiment repetitions. A preweighed amount of 0.1 g of clay-based microsclerotial granules was added to the surface of each Petri dish and incubated at 26 °C for 1 week. Then the number of conidia produced was determined by hemocytometer counts from conidia suspensions prepared by adding 3 ml of 0.05% Tween-80. Percent germination of the conidia was assessed by spreading a 100 µl aliquot of each sample onto each of two 6-cm diameter Petri dishes containing half strength Sabouraud dextrose agar with yeast (SDAY). Dishes were sealed with parafilm and incubated 16–20 h at 26 ± 1°C. The first 100–200 conidia observed at 400× will were scored for germination at two arbitrary locations for each dish.

Results. In the first experiment, chlorothalonil did not inhibit the growth of the fungus; iprodione showed slight inhibitory effect at higher concentration; propiconazole, twinline and stratego strongly inhibit the fungal growth except at the lowest concentration of 1 mg/L. The second and third experiments had similar results to the first experiment, except that iprodione only suppressed fungal growth at the highest concentration of 1000 mg/L. This was probably due to the degradation of iprodione in the fungicide-infused agar medium.

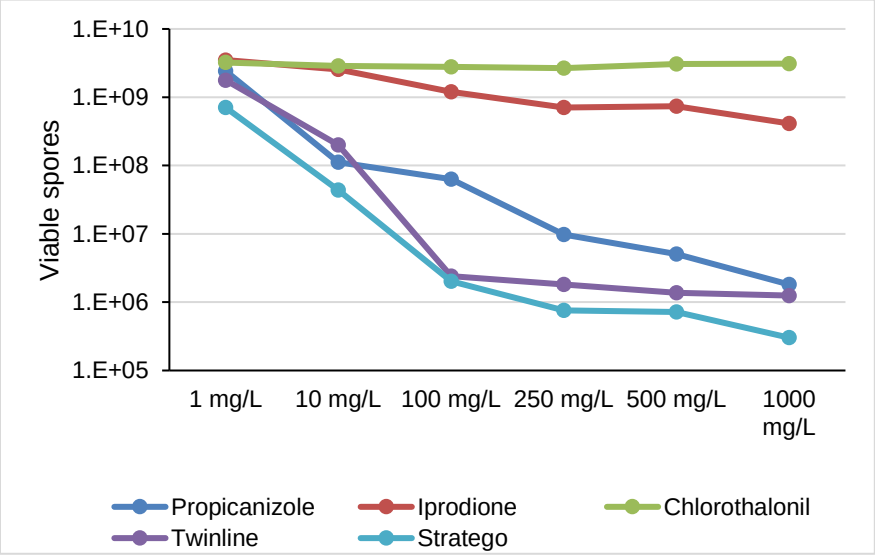

Fig. 1. Number of viable spores per gram of *Metarhizium brunneum* microsclerotia in treatments with different fungicides in different concentrations.

Greenhouse tests

Methods. Based on our laboratory findings, three fungicides commonly used in turfgrass, were used to test the effect on colony forming units (CFUs) produced from *M. brunneum* microsclerotia in grass pots under greenhouse conditions using a low and a high rate from the labeled range. The rates were 0.5 and 1.8 kg ai/ha for propiconazole (Banner Max® II, 14.3% A.I.); 1.5 and 6 kg ai/ha for Iprodione (Chipco 26GT®, 23.3% A.I.); 2.2 and 8.6 kg ai/ha for chlorothalonil (Daconil Weather Stik®, 54.0% A.I.). Microsclerotia was applied at 20 mg/pot (20 kg/ha, 1.45×109 CFUs/g) to pots with creeping bentgrass. In addition, a blank control without fungicide treatments was used. The fungicides were applied 24 h after the fungus using a Generation III Research sprayer with a spray volume of 748 L/ha. The pots were watered well 1 d before fungal application, and received light irrigation (3 mm) to wash the microsclerotia granules onto the substrate surface, and then irrigated with 7 mm water every other day to provide moisture for microsclerotia and grass growth. Each treatment was replicated in three grass pots, and the experiment was conducted three times.

Pots were evaluated at 10, 20 and 30 days after treatment by determining the number of CFUs recovered in each pot. The top 2.5 cm of soil and the grass were collected in bags and brought to the laboratory to determine conidial spore concentrations in the soil. The contents of the bags were mixed thoroughly. Two 5-g samples were taken from each pot and diluted with 50 ml sterile 0.05% Tween20 solution and shaken vigorously by hand to ensure a homogenous suspension for 5 minutes. Following 5 seconds sedimentation, 1 ml of the soil suspension was transferred to 9 ml 0.05% Tween20 to make 10% dilution. The diluted suspension was vortexed for 1 min with several 3 mm glass beads, and 0.1 ml suspension was removed from the surface layer and spread onto each of two PDAY 0.015% dodine media plates (Rangel et al. 2010). Plates were incubated at 26 ± 1 °C on a 12 h photoperiod. After 7 days, the number of colonies appearing on the plates were counted and the CFUs/pot were determined. The moisture content of each soil sample was determined by drying a pre-weighed quantity of collected soil in an oven for 3 d at 72 °C and then air-drying for another 3 d.

Two-way ANOVA was used to determine differences between treatments and between evaluation times at a significance level of 0.05. Data were log transformed before analysis.

Results. Significant differences were found between treatments ($F = 8.20$; df = 6, 187; $P < 0.0001$). Propiconazole had the lowest number of CFUs and its high rate significantly inhibited fungal growth. The CFUs in other treatments were not significantly different from the untreated control (Tukey's test, $\alpha = 0.05$). There was no effect of evaluation time ($P = 0.127$) and no significant interactions between treatment and evaluation date ($P = 0.055$).

Obj. 2. Efficacy of formulation against ABW adults and externally feeding larvae.

Efficacy vs. adults

Methods. The efficacy of *M. brunneum* microsclerotia against ABW adults was evaluated in pots with creeping bentgrass under greenhouse conditions. Microsclerotia granules were applied at 0, 10, 20 and 40 mg/pot (equal to 0, 11.5, 23 and 46 kg/ha) 10 d before adding adults. There were six replciates per treatment in randomized block design and the experiment was conducted twice. In each pot, 10 weevils were caged with mesh lids, and dead and live weevils were recovered by soaking in warm water for 20 min at 14 d after weevil introduction. The

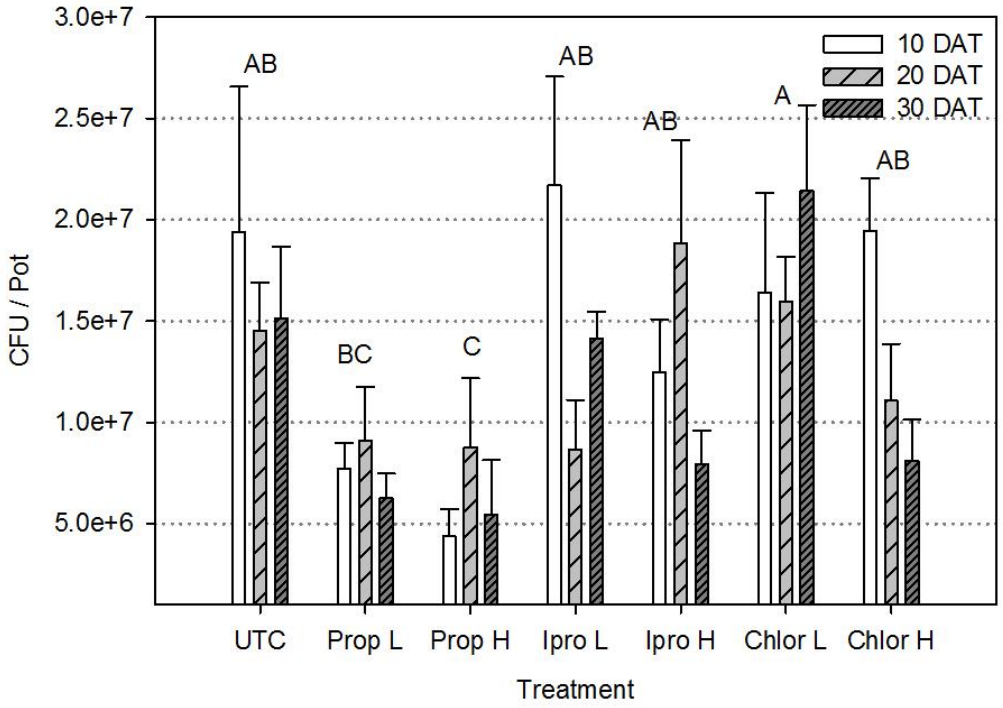

Fig. 2. Number of *Metarhizium brunneum* colony forming units (CFUs) recovered in grass pots treated with water only (untreated control = UTC), and a low (L) and a high (H) rate of the fungicides propiconazole (Prop), iprodione (Ipro) and chlorothalonil (Chlor) at 10, 20 and 30 days after treatment (DAT). Means with same letter did not differ significantly from each other Different letters indicate significant difference between treatments (data for evaluation dates combined) (Tukey's test, α=0.05).

recovered weevils were brought to the lab to incubate for 3 d to observe potential fungal growth. The pots were watered well 1 d before fungal application, and received light irrigation (3 mm) to wash the microsclerotia granules onto the substrate surface, and then irrigated with 7 mm water every other day to provide moisture for microsclerotia and grass growth.

Weevil mortality was calculated with the formula: Mortality = 100 × (N – live number)/N, N was the total number of weevils released in each pot before treatment. One-way ANOVA was used to detect the significant difference among treatments at α=0.05.

Results. No difference was found between various doses of *M. brunneum* microsclerotia ($F = 0.26$; df = 3, 46; $P = 0.86$), and the fungal treatments did not have significantly higher weevil mortality than the untreated control. A few adults were infected with the fungus in each treatment with the microsclerotia; however, the rate was too low to achieve a significant level of control.

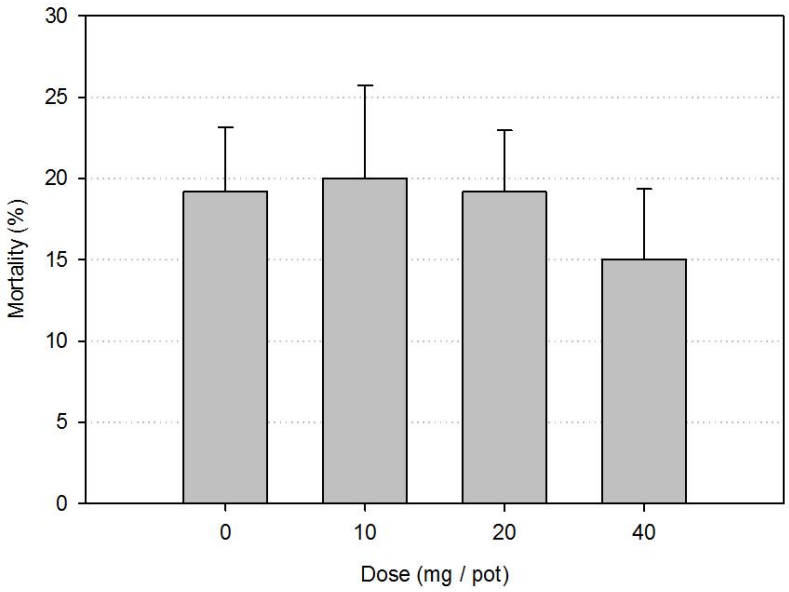

Fig. 3. Mortality of ABW adults exposed to different doses of *Metarhizium brunneum* microsclerotia under greenhouse conditions (mean ± sem).

Efficacy vs. larvae

Methods. *Poa annua* pots with developing ABW larvae (see above) were treated with microsclerotia granules at 2.5, 5, 10, and 20 mg per pot at 2 weeks after adult removal which is 1 week before the larvae are expected to be on average in the fourth stage. At 5 weeks after adult removal, the number of surviving ABW stages were determined by careful manual examination of soil and grass followed by submersion in saturated salt solution. There were five replications per treatment in each of 2 experiment repetitions.

Results. In the first experiment, no significant suppression of larvae was observed with no more than 25% lower larval densities than in the untreated control. The second experiment was still ongoing at the time of this report.

Experiments comparing pyrethroid-resistant and susceptible ABW larvae and comparison with granular and liquid commercial Met52 formulations still need to be conducted. The commercial formulation were not available at this time since the company had temporarily stopped production but will resume soon due to popular demand.

Obj. 3. Dissemination of generated knowledge to the golf course industry.

Our findings are disseminated to the golf course industry through our regular extension activities including (1) oral presentations and demonstrations at field days, regional and national turf conferences and trade shows, etc., including NYSTA educational events such as Southeast Regional Conference in Fishkill, NY, (2) publications in regional and national trade journals, (3) articles in newsletters including the NYSTA e-newsletter and on turf blogs, (4) extension fact sheets and bulletins, and (5) webpages including the NYSTA webpage. Dr. Koppenhöfer is involved in these outreach activities in New York and neighboring states. Furthermore, our findings will be published in scientific journals and presented at regional and national scientific meetings. Specifically with regards to NYSTA and New York state, Dr. Koppenhöfer gave a presentations at NYSTA Southeast Regional Conference (1-26-2017, Fishkill, NY) on 'Angry

annual bluegrass weevil: insecticide resistance in ABW, how to deal with it, and how to avoid it' and at the MetGCSA / CAGS Educ. Sem. (11-17-2016, Fairfield, CT) on 'Don't get them angry: Avoiding and managing insecticide resistance in ABW.' Findings of the previous TESF grant were published in the NYSTA Electronic Newsletter (**Koppenhöfer,** Kostromytska, Wu. 2015. Insecticide resistant annual bluegrass weevil: Understanding, managing, and preventing a superintendent's nightmare. NY State Turf Assoc. Electronic Newsletter, December 2015.).

Conclusions to date and plans for the future

Our lab and greenhouse studies with fungicides showed that *M. brunneum* F52 microsclerotia use is compatible with fungicide use on golf courses. Only propiconazole had some suppressing effect. Under field conditions, any negative effects would likely be further reduced.

Under laboratory conditions in Petri dishes on moist soil the fungus (data not shown) killed high percentages of adults. But this did not occur under greenhouse conditions. Since we provided sufficient moisture in the greenhouse, we did not include hydrogel into the treatments under greenhouse conditions. Hence, be believe that the adult ABW is not a good target for the fungus, especially in the spring when temperature tend to be cooler.

In the first greenhouse experiment with larvae only limited mortality of larvae by the fungus was observed. We have no reason to believe that there will be dramatically higher control in the second ongoing experiment. Unfortunately, we have no efficient method to work with ABW larvae in the laboratory. However, we believe that combining *M. brunneum* with a neonicotinoid insecticide, especially imidacloprid, has potential to result in synergistic mortality as has been observed with other insect and fungus-imidacloprid combinations. Such a combination treatment, could not only control ABW larvae, but would at the same time control white grubs later in the year. We are therefore planning to conduct a field experiment this spring that will include *M. brunneum* microsclerotia granules alone and in combinations with hydrogel and / or imidacloprid. Should these results be promising, we will continue our studies with the fungus with additional greenhouse experiments in early 2018 and potentially field experiments in spring of 2018.

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