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Cover crop and conidia delivery system impacts on soil persistence of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) in sugarbeet

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The sugarbeet root maggot, *Tetanops myopaeformis* (Röder), is a major North American pest of sugarbeet, *Beta vulgaris* L. Previous research suggests that moderate *T. myopaeformis* control is possible with the entomopathogen *Metarhizium anisopliae* (Metch.) Sorok. We conducted a three-year (2002–2004) experiment to assess impacts of oat, *Avena sativa* L. and rye, *Secale cereale* L., cover crops on persistence of corn grit-based granular or spray formulations of *M. anisopliae* isolate ATCC 62176 (i.e. MA 1200) applied at 8×10^{12} viable conidia/ha in sugarbeet. More colony forming units (CFUs) were detected immediately after application [0 days after treatment (DAT)] in spray plots than granule-treated plots. However, 76–92% declines in CFUs per gram of soil occurred in spray plots within 30 DAT. Substantially (i.e. 83–560%) more rainfall occurred in June 2002 than during June of any other year. Subsequently, 71–670% increases in CFU concentrations occurred by 60 DAT in *M. anisopliae* granule-treated plots with oat or rye cover crops that year. CFU density increases were higher in cover crops in 2002, but no significant cover crop effects were detected. Conidia persisted for up to 30 DAT in *M. anisopliae* spray plots and 60 DAT in granule-treated plots in 2002; however, no increases occurred in the years with less June rainfall. Trends suggest that *M. anisopliae* aqueous sprays result in greater conidia concentrations than granules at sugarbeet plant bases in June during *T. myopaeformis* oviposition and larval establishment on host plants. Increases are possible when delivering conidia via granules, but high post-application rainfall could be necessary for conidia production.

Keywords: *Tetanops myopaeformis*; entomopathogenic fungus; insect pathogen; microbial insecticide; soil moisture; integrated control

1. Introduction

The sugarbeet root maggot, *Tetanops myopaeformis* (Röder) (Diptera: Ulidiidae), is the most important insect pest of sugarbeet, *Beta vulgaris* L., in the Red River Valley of North Dakota and Minnesota, USA. It is also a significant threat to the crop in several western states and the Canadian province of Alberta. Larvae of *T. myopaeformis* feed by scraping root surfaces with paired oral hooks and consuming the exudate from feeding sites. Severe feeding injury can result in seedling death if the

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tap root becomes severed, especially under extremely dry soil conditions. Sugarbeet yield losses resulting from *T. myopaeformis* feeding injury can exceed 40% in the absence of adequate control measures (Campbell, Anderson, Dregseth, and Smith 1998; Boetel, Dregseth, and Schroeder 2010). Dryland (i.e. non-irrigated) sugarbeet fields at risk of economically damaging *T. myopaeformis* infestations are often prophylactically treated at planting with a soil-applied granular or liquid chemical insecticide, or an insecticidal seed treatment (Peay, Beards, and Swenson 1969; Yun and Sullivan 1980; Bergen 1984; Bergen, Whitfield, and Lilly 1986; Carlson, Boetel, Khan, and Stachler 2012). Granules are delivered in 5- to 18-cm swaths over the row through conventional banders, in-furrow tubes, or 'spoon' placement devices (Boetel, Dregseth, Schroeder, and Doetkott 2006). Supplementary mid-season applications of either a banded granular material or a liquid chemical insecticide (i.e. banded or broadcast delivery) are also frequently used to protect the crop from moderately high to severe *T. myopaeformis* infestations that frequently develop in the Red River Valley production area. Post-emergence granular applications are targeted at the larval stage of the pest, whereas liquid formulations are aimed at controlling both adults and larvae. Alternative *T. myopaeformis* control methods would be critically needed if federal registrations of conventional chemical insecticides were cancelled as a result of regulatory action relating to environmental concerns, or if populations were to develop insecticide resistance due to the chronic use of synthetic chemical insecticides to control this pest during the past few decades.

Smith (1990) was the first to strongly advocate the development of a biocontrol programme for *T. myopaeformis* management. Smith and Eide (1995) conducted laboratory assays with isolate ATCC 22099 of the entomopathogenic fungus *Metarhizium anisopliae* (Metch.) Sorok., and reported high virulence to *T. myopaeformis* larvae. Campbell, Boetel, Jonason, Jaronski, and Smith (2006) tested the efficacy of granular and post-emergence aqueous spray formulations of *M. anisopliae* isolate ATCC 62176 (i.e. MA 1200) on *T. myopaeformis* under field conditions and suggested conidial concentration, application timing, and soil moisture as the most important determinants for success in using this fungus for *T. myopaeformis* management. The principle underlying use of entomopathogenic fungi on nutritive granules at planting is to create a zone of conidia in the furrow placed to intercept neonate larvae migrating to the tap root. Post-emergence aqueous spray applications of conidia to plant bases are aimed at saturating the very upper (i.e. top 0.5 cm) layer of soil where females deposit eggs; hatching larvae then emerge into the conidia-treated target zone. In either case, the goal is for larvae to acquire an infectious dose as they pass through the soil during host finding and establishment. Campbell et al. (2006) suggested that commercial formulations should be designed for application with conventional equipment to facilitate adoption of fungus-based control tools by sugarbeet growers.

Persistence and consistency of formulations also have been suggested as important features of a biopesticide for insect control in sugarbeet (Campbell, Eide, and Jonason 2000; Jaronski, Fuller-Schaeffer, Jung, Boetel, and Majumdar 2007). Obviously, an efficacious concentration of live conidia in the path of larvae is critically important for insect control, and a number of factors can affect the viability of conidia in soil (Jaronski 2007). The association of environmental effects with persistence of *M. anisopliae* conidia has long been a topic of research. In a review on entomopathogenic Ascomycete soil ecology, Jaronski (2007) found that there is

typically an inverse relationship between temperature and persistence of entomopathogenic fungus conidia, whether in a stored formulation or in the environment; however, exceptions are also apparent. Clerk and Madelin (1965) suggested temperatures in the range of 8–25°C as adequate for survival of *M. anisopliae* conidia, and further indicated that 45% relative humidity is optimal for the fungus. Conversely, Daoust and Roberts (1983) observed high survival of *M. anisopliae* conidia at the extremes of 0 and 100% humidity, and reduced shelf life in storage at intermediate humidity levels. Their findings were somewhat supportive of Walstad, Anderson, and Stambaugh (1970) who reported temperatures within a range of 15–35°C and humidity exceeding 92% to be optimal for *M. anisopliae.* However, as pointed out by Jaronski (2007), considerable variability exists among fungal isolates in response to temperature. Thus, overgeneralisation among species or across isolates within species in relation to environmental impacts on persistence of entomopathogenic fungi should be avoided.

According to Hallsworth and Magan (1999), optimal relative humidity in the soil microenvironment for growth and development of *M. anisopliae* varies from 97 to 99%. Vänninen (1995) studied the effect of location, habitat, and soil type on survival of entomopathogens, and reported that *M. anisopliae* conidia were typically resistant to biodegradation and capable of long-term survival in cultivated habitats; however, conidia became non-viable when the soil temperature fell below 10°C. Bing and Lewis (1993) and Hummel et al. (2002) found conservation tillage and no-till microhabitats to be more conducive to the persistence of *M. anisopliae* conidia in soil than habitats established by using conventional tillage.

In recent years, there has been considerable interest in the phenomenon of rhizosphere (i.e. root/soil interface) colonisation by entomopathogenic fungi and its implications for fungal survival and sporulation (Hu and St. Leger 2002; Klingen, Hajek, Meadow, and Renwick 2002; Bruck 2005, 2010). In one experiment, Bruck (2005) observed 80% control of black vine weevil, *Otiorhynchus sulcatus* (F.), larvae within two weeks of exposure to *Picea abies* (L.) roots colonised by *M. anisopliae.* The pursuit of fungal strains that are virulent to the target pest, efficient at colonising the rhizosphere, and exhibit prolonged persistence could provide excellent candidates for future use as mycoinsecticides for managing soil-dwelling insect pests. The negative effects of soil microflora, either indirectly through interspecific competition or directly by way of antibiosis, have been suggested as important limiting factors that can compromise the success of entomopathogenic fungi under field conditions (Lingg and Donaldson 1981; Inglis, Goettel, Butt, and Strasser 2001). The deleterious impacts of solar radiation also have been observed by multiple authors as limiting factors to the successful deployment of entomopathogenic fungi in both field and laboratory settings (Gardner, Sutton, and Noblet 1977; Ignoffo, Hostetter, Sikorowski, Sutter, and Brooks 1977; Inglis, Goettel, and Johnson 1993, 1995; Hunt, Moore, Higgins, and Prior 1994; Smits, Fargues, Rougier, Goujet, and Itier 1996; Inglis, Johnson, and Goettel 1997; Alves, Bateman, Prior, and Leather 1998; Braga, Flint, Miller, Anderson, and Roberts 2001a,b; Lee et al. 2002; Behle 2006).

Cereal cover crops have been shown to provide the following agronomic benefits in sugarbeet: protection of seedlings from wind damage (Fornstrom and Miller 1996); increased soil stability (Sommer and Schwerdtle 1984); and improved soil moisture retention (Fornstrom and Miller 1996). Other research has demonstrated that cover crops can serve as cultural tools for pest management in sugarbeet

production systems, specifically in relation to either weed (Fornstrom and Miller 1996) or *T. myopaeformis* management (Dregseth, Boetel, Schroeder, Carlson, and Armstrong 2003). The success of an oat, *Avena sativa* L., cover crop as a cultural tool for *T. myopaeformis* control was speculated as resulting from either conservation of soil moisture that could positively impact release of active ingredient from the granular carrier of a conventional organophosphate insecticide (e.g. terbufos), or greater exposure of larvae to insecticides in the treated zone due to modified larval behaviour in the cover crop rhizoplane microhabitat (Dregseth et al. 2003). It is possible that cover crops also could modulate the soil microenvironment to increase the efficacy of an entomopathogenic fungus such as *M. anisopliae*. The objective of this research was to assess the impacts of oat and rye, *Secale cereale* L., cover crop microhabitats on field persistence of *M. anisopliae* conidia applied to sugarbeet in dryland (i.e. non-irrigated) production systems. An additional objective was to determine the impact of delivery system (i.e. planting-time-applied corn grit-based granules or post-emergence aqueous sprays) on soil persistence of the fungus.

2. Materials and methods

2.1. Field plot methodology

Field studies were conducted in 2002, 2003 and 2004 near St. Thomas (Pembina County, ND, USA), an area that is perennially infested with high *T. myopaeformis* populations. A randomised complete block design (RCBD) with four replications was used in all three years of the experiment. In 2002 and 2003, a split-plot arrangement was used with cover crop seeding rate serving as the main-plot factor and fungus delivery system (i.e. *M. anisopliae* conidia via granular or liquid carrier and an untreated check plot) as the subplot factor. In 2004, a split-split plot arrangement of the following was established: cover crop type (i.e. oat or rye) as the main-plot factor, seeding rate as the subplot factor and insecticide regime (as described earlier) served as the sub-subplot factor. Plots were established on soils possessing the following characteristics: (1) 2002: Bearden silt loam, 5.3% organic matter, 7.8 pH; (2) 2003: Glyndon silt loam, 4.0% organic matter, 7.9 pH; and (3) 2004: Neche silty clay loam, 3.5% organic matter, 8.0 pH.

Cover crops were planted according to the procedures of Dregseth et al. (2003). Cultivars used included 'Newdak' oat, 'Dacold' rye and VanderHave 66240 sugarbeet, and the same cultivars were used each year. In 2002 and 2003, cover crops were sown on a volumetric basis at 0, 131 and 262 L seeds/ha (i.e. rates commonly used by sugarbeet growers in the region). In 2004, cover crop seeding rates were adjusted to establish the same number of plants per unit area, and were planted at 0, 186 and 374 seeds/m^2. Plots were 10.7 m long by 3.3 m wide (i.e. six sugarbeet rows, spaced 0.56 m apart) in all study years. The two outer rows of adjacent plots served as untreated buffer rows between plots. Cover crop seeds were broadcast-sown in salt-shaker fashion by using clean 591-ml beverage containers with 0.8- or 1.0-cm diametre holes in the bottoms for delivery of rye and oat seed, respectively. Seeds were lightly incorporated into the upper 2 cm of soil by using a motorised walk-behind garden tiller immediately after seeding. Sugarbeet was planted immediately after incorporation of cover crop seed. Sethoxydim (Poast™, BASF Corporation, Research Triangle Park, NC, USA) herbicide was applied to cover crop plots at 0.22

kg (AI)/ha between 7 and 9 June each study year to slow cover crop growth and minimise interplant competition with sugarbeet plants. A second application of the herbicide was made about one week later each year at 0.45 kg (AI)/ha to kill the oat and rye plants when extended leaf length was about 15 cm.

2.2. M. anisopliae *formulations*

Conidia of *M. anisopliae* isolate ATCC 62176 for both granular and aqueous spray applications in this experiment were mass-produced at the USDA-ARS Northern Plains Agricultural Research Laboratory (Sidney, MT, USA). The identity of ATCC 62176 was confirmed as an isolate of *M. anisopliae* by sequencing the 5′ end of translation elongation factor 1-alpha gene as per Bischoff, Rehner, and Humber (2009). The resulting sequence was deposited in GenBank (Benson, Karsch-Mizrachi, Lipman, Ostell, and Wheeler 2005) as accession number JQ585929. Sequential, diphasic liquid–solid fermentation methodology, based on that of Bradley, Wood, Black, Kearns, and Britton (2002), was used for all conidia production in each study year. This initially involved re-isolating it from infected *T. myopaeformis* larvae. Conidia, subcultured twice on half-strength Sabouraud dextrose agar medium plus 0.1% yeast extract were then used as primary inoculum for initial subculturing on liquid and subsequently on solid media. The liquid fermentation phase involved a fluid medium that was inoculated with the primary culture of *M. anisopliae* and incubated for 3–4 d at 25 ± 1°C. Liquid cultures containing blastospores were added to autoclaved hydrated flaked barley, *Hordeum vulgare* L., (Minnesota Grain, Inc., Eden, MN, USA) in sterile SacO2™ mushroom spawn bags (Microsac, Eke, Belgium) for conidia production. The solid fermentation phase was carried out for eight days under constant darkness at 25 ± 1°C. The resulting culture was air dried for seven days at 24 ± 1°C and 30–40% RH. Conidia for both granular and spray formulations were harvested by running the entire dried solid culture through 20- and 100-mesh ASTM-grade (American Society for Testing and Materials) screens mounted on a vibratory shaker to yield a powder consisting of $4–6 \times 10^{10}$ conidia/g at a moisture content of $<5\%$ (w/w). Conidia viability ($>95\%$) was assessed by observing germination on yeast extract agar (Sigma-Aldrich, St. Louis, MO, USA) amended with 0.005% benomyl (Sigma-Aldrich) after 16–20 h of incubation at 27 ± 1°C.

For the granular formulation, dried conidia were coated onto 16- to 20-mesh (i.e. 0.5–1 mm diam.) corn grit granules (Bunge North America, Inc., St Louis, MO, USA) using 20% aqueous monosorbitan oleate (Tween™ 20, Sigma-Aldrich) as a binder to produce a formulation with a titre of 3.6×10^{11} viable conidia/kg (ca. 2.5×10^{5} conidia per granule). Corn grit was first coated with the Tween binder by spraying the liquid using an airbrush and mixing in a conventional V-cone blender for 10 min. The requisite amount of conidia was then dusted onto the slightly sticky carrier, and the combination was vigorously mixed in the blender. Any remaining stickiness of the granules was eliminated by dusting the material with unscented talcum powder and blending for an additional 5–10 min. Formulated granules and conidia for spray treatments were stored and transported under dry refrigeration at 5 ± 1°C until use and applied within 48–72 h of preparation each year. Freshly prepared fungus formulations were used during each year of the experiment. Preliminary stability studies conducted in the process of developing these granules

indicated that the conidia did not lose viability under these conditions (S.T. Jaronski, unpublished data).

2.3. Conidia applications

Each fungus formulation was applied at 8.0×10^{12} viable conidia/ha in all study years. The granular formulation was applied at 22 kg/ha in mid- to late-May of each year at sugarbeet planting by using modified in-furrow placement (Boetel et al. 2006), which places granules in a narrow (5-cm) swath over the row and into the upper portion of the seed furrow, just above the seed level. This technique is commonly used by growers for prophylactic applications of conventional chemical insecticides to manage *T. myopaeformis* larvae that typically begin feeding on sugarbeet roots about six weeks after planting. A commercial John Deere™ 71 Flex six-row planter (Deere & Company, Moline, IL, USA) was used to plant all plots. Planter-mounted Noble™ (Remcor, Howe, TX, USA) metering units were used to regulate granular delivery rates, and all units were calibrated on the planter before treatment applications.

Post-emergence liquid spray applications were made during peak *T. myopaeformis* fly activity (i.e. mid- to late-June) of each study year to target adults and neonate larvae at sugarbeet plant bases. The applications consisted of conidial suspensions in 0.1% aqueous monosorbitan oleate (Tween™ 80, Sigma-Aldrich). Preparation of spore suspensions was based on conidial concentration and viability in each year's conidial powder. The requisite amount of conidial powder was first suspended in 1–2 L of 1.0% Tween 80, and added to the remaining water. The suspension containing conidia was vigorously hand-shaken immediately before and throughout applications to maintain uniformity of the spray mixture and ensure consistent delivery of the intended rate of conidia during the application process. Conidial viability was assessed on a sample of the sprayed suspension each year, and found to be consistently $>90\%$. Post-emergence sprays were applied in 18-cm bands over sugarbeet rows by using a CO_2-propelled sprayer system equipped with Teejet™ 6503E nozzles (Teejet Technologies, Wheaton, IL, USA). All spray treatments were delivered in an output volume of 280 L/ha. Sugarbeet seedlings were in the 4- to 6-leaf stage at the time of all post-emergence applications.

2.4. Post application conidia recovery

Soil samples were collected at 0 d (i.e. immediately after treatment) and 30 or 60 days after treatment (DAT) to assess fungal persistence. In 2002, soil sampling for post-application persistence assessments were conducted at 60 d after planting, which was 60 DAT for the granular fungus formulation and 30 DAT for the post-emergence aqueous spray applications. In 2003 and 2004, soil sampling for conidia persistence in both granular and spray applications was conducted at the same post-application interval (i.e. 30 DAT). At each sampling, a stainless steel soil core sampler (5 cm diam.) was used to collect two soil samples from each of the outer two treated rows of each plot (i.e. four total samples per plot) by carefully placing the device over the centre of the treated zone and driving it downward to a depth of 4 cm. A clean core sampler was used between all treatments at each sampling date to prevent cross-contamination of soil samples, and sample sites within each row were marked with a

wire flag at each date to prevent repeated sampling of the same soil. Immediately following collection, soil samples were deposited into clean Ziploc™ (Racine, WI, USA) resealable plastic bags and placed into a dry cooler at $20 \pm 1°C$, and transported to the laboratory where they were stored at 5°C pending laboratory processing.

Dilution plating was used to assess viability of *M. anisopliae* conidia present in soil samples. All samples were mixed thoroughly to ensure homogeneity of conidial concentration per unit of soil before subsamples were drawn. Two subsamples, 1 g for dilution plating (i.e. viability testing) and 2 g for soil moisture quantification, were collected from each composite sample. Each 1-g soil sample collected for viability testing was suspended in 9 ml of sterile water containing 0.1% monosorbitan oleate (Tween™ 20, Sigma-Aldrich). The suspension was sonicated for 15 min to breakup soil clumps. After sonication, 1 ml of the suspension was serially diluted to a 10^{-2} dilution. Aliquots of 100 μl each were spread onto four Petri plates containing 20 ml of modified Chase medium [i.e. 20 g/L oatmeal, 20 g/L agar, 0.6 g/L dodine, 1 ml/L gentamycin and 0.001 g/L crystal violet (Chase, Osborne, and Ferguson 1986)]. Distinctly green circular colonies of *M. anisopliae* formed on the blue medium. Colony forming units (CFUs) were counted after 10 d of incubation at $25 \pm 1°C$. The 2-g soil samples were dried at $65 \pm 1°C$ for 48 h and reweighed to determine moisture content. The CFU counts from dilution plates were then adjusted to obtain final concentrations of CFUs per gram of dry soil.

2.5. Soil temperature and moisture monitoring

Soil temperature and soil water tension, a measurement of available soil water, were monitored continuously in all plots, including the untreated (i.e. no cover crop) controls. Temperature was monitored by using WatchDog™ (Model 425, Spectrum Technologies, Inc., Plainfield, IL, USA) data loggers. Deployment of data loggers involved placing them inside manufacturer-provided heat/radiation shields, mounting them on a metal fence post by using 2 U-shaped metal bolts and positioning them in the centres of pre-assigned plots. Additional environmental monitoring included placement of a Watermark™ (Spectrum Technologies) soil water tension sensor and a soil temperature probe at seeding depth (i.e. 3 cm below surface) within a plot row, and burying them to ground level with field soil. This placement resulted in probes being positioned in immediate proximity to planting time-applied fungus granules. Pre-calibrated sensors recorded observations every two hours, and data were retrieved at the end of each growing season by using the manufacturer-provided SpecWare v. 6.0 (Spectrum Technologies) software.

2.6. Data analysis

Mean CFU counts per gram of dry soil from each treatment were subjected to analysis of variance (PROC ANOVA, SAS Institute 1999) using a model appropriate for the respective experimental designs. The 2002 and 2003 data were analysed as an RCBD with a split plot in time arrangement, and data from 2004 were analysed as an RCBD with a split-split plot in time arrangement. A folded *F*-test (Steel, Torrie, and Dickey 1997) was conducted by using error mean sums of squares to determine the feasibility of a combined analysis for the 2002 and 2003 data because seeding rates

were identical for those years. Treatment means from data sets generating significant interaction terms were separated by using Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$.

3. Results

3.1. Conidia survival

ANOVA indicated that data from 2002 to 2003 could not be combined because error mean squares from the initial folded *F*-test were significantly ($P < 0.05$) different. Results from the ANOVAs for 2002 and 2003 are presented by year in Table 1. In both years, cover crop type had no significant effect ($P = 0.3474$) on survival of *M. anisopliae* conidia; however, formulation significantly affected conidia persistence in both 2002 ($P < 0.0001$) and 2003 ($P < 0.0001$). Sampling date also had a significant impact on persistence in the first two study years (2002: $P = 0.0110$; and 2003: $P = 0.0254$). Significant formulation $\times$ sampling date interactions also were detected for 2002 ($P < 0.0001$) and 2003 ($P < 0.0001$).

The ANOVA for 2004 persistence data is provided in Table 2. As was observed for the first two years of this experiment, significant impacts of formulation ($P < 0.0001$), sampling date ($P = 0.0035$) were also detected in 2004. Also corresponding to the results of 2002 and 2003 was a significant formulation $\times$ sampling date interaction ($P < 0.0001$) in 2004. As observed in the first two years of the experiment, there was no effect of cover crop type on conidial viability in 2004. Additionally, there was no significant impact ($P = 0.7169$) of seeding rate on survival of *M. anisopliae* conidia in 2004.

Mean CFU counts from soil sampling procedures are summarised for all study years in Table 3. Soil samples collected immediately after applications (0 DAT) indicated that *M. anisopliae* spray applications resulted in significantly ($P < 0.05$) higher concentrations of CFUs per gram of soil immediately adjacent to sugarbeet plant bases than granular applications, irrespective of whether a cover crop was present. However, major (i.e. 81.6–92%) reductions in CFU concentrations occurred in fungus spray-treated plots within 30 DAT. Losses in viable conidia appeared to be slightly lower in spray-treated plots when oat cover crops were established at the low seeding rate, although no significant differences were detected between cover crop types or seeding rates.

Large (i.e. 71–670%) increases in CFU densities per gram of soil were observed between 0 and 60 DAT in plots treated with the granular formulation of *M. anisopliae* in 2002; however, high variability among samples precluded the detection of statistically significant differences between sampling dates. No further CFU increases were detected for liquid or granular applications in subsequent study years. General trends from samples collected at 30 DAT in 2003 and 2004, years in which sampling dates were identical for both formulations, indicated that *M. anisopliae* conidia applied by using aqueous spray formulations tended to survive better in cover crop microenvironments (mean = 1470 CFUs/g soil) than those applied to plots lacking a cover crop (mean = 997 CFUs/g).

Persistence data were pooled for cover crop type and seeding rate within years, and are graphically depicted in Figure 1. Results of that treatment of the data indicated that spray applications resulted in high initial CFU concentrations immediately after

Table 1. Analysis of variance for effects of cover crop type, fungus formulation, and sampling date on *Metarhizium anisopliae* spore persistence in sugarbeet, St. Thomas, ND, USA, 2002–2003.

		2002			2003		
Source	df	Mean square	*F*	*P*	Mean square	*F*	*P*
Replication	3	1051.809	0.95	0.4286	219.123	1.38	0.2693
Cover type[a]	4	884.037	0.74	0.5850	159.059	1.24	0.3474
Error (a) (replication × cover type)	12	1201.300	–	–	128.788	–	–
Formulation[b]	1	58639.035	45.41	<0.0001	5824.284	45.93	<0.0001
Cover type × formulation	4	1045.222	0.81	0.5383	149.918	1.18	0.3583
Error (b) (replication × cover type × formulation)	15	1291.292	–	–	126.811	–	–
Sampling date[c]	1	42610.296	31.87	0.0110	4360.104	17.25	0.0254
Error (c) (replication × sampling date)	3	1337.127	–	–	252.688	–	–
Cover type × sampling date	4	1118.789	1.01	0.4174	132.279	0.83	0.5150
Formulation × sampling date	1	58379.415	52.94	<0.0001	3931.208	24.81	<0.0001
Cover type × formulation × sampling date	4	773.560	0.70	0.5977	129.339	0.82	0.5260
Error (d)	27	1102.7245	–	–	158.462	–	–

[a]Oat, *Avena sativa* L. and rye, *Secale cereale* L.
[b]Conidia were applied by using granular or aqueous spray formulations.
[c]0, 30 or 60 DAT.

Table 2. Analysis of variance for effects of cover crop type, seeding rate, fungus formulation, and sampling date on *Metarhizium anisopliae* spore persistence in sugarbeet, St. Thomas, ND, USA, 2004.

Source	df	Mean square	F	P
Replication	3	16791.945	1.39	0.2682
Cover type[a]	1	83.364	0.01	0.9152
Error (a) (replication × cover type)	3	6226.715	–	–
Seeding rate[b]	2	4552.400	0.35	0.7169
Cover type × seeding rate	1	7070.494	0.54	0.4826
Error (b) (replication × cover type × seeding rate)	9	13180.971	–	–
Formulation[c]	1	1387151.420	145.50	$<$0.0001
Cover type × formulation	1	573.018	0.06	0.8097
Seeding rate × formulation	2	3449.191	0.36	0.7023
Cover type × seeding rate × formulation	1	3990.279	0.42	0.5274
Error (c) (replication × cover type × seeding rate × formulation)	15	11073.077	–	–
Sampling date[d]	1	1093204.911	70.67	0.0035
Error (d) (replication × sampling date)	3	15468.986	–	–
Cover type × sampling date	1	1545.637	0.13	0.7235
Seeding rate × sampling date	2	3509.930	0.29	0.7503
Formulation × sampling date	1	1222780.369	101.18	$<$0.0001
Error (e)	26	12085.752	–	–

[a]Oat, *Avena sativa* L. and rye, *Secale cereale* L.
[b]Cover crops were sown at 0, 186 and 374 seeds/m^2.
[c]Conidia were applied by using granular or aqueous spray formulations.
[d]0, 30, 60 DAT.

applications (i.e. 0 DAT). Marked decreases were observed in fungus spray plots at 30 DAT in all study years, but there was no significant difference in CFU counts per gram of dry soil between the 30 DAT assessments of spray-applied conidia and either 30 or 60 DAT counts from granule-treated plots. Conidial concentrations underwent a large (129%) numerical increase between 0 and 60 DAT in plots treated with the granular formulation in 2002; however, high variability prevented detection of a statistical difference in conidia counts between sampling dates.

3.2. Effect of cover crops on soil microclimate

Soil water tension at sugarbeet seeding depth (i.e. 3 cm) appeared to be moderated by both cover crop type and seeding rate (Table 4). In 2002 and 2003, plots established with the high (i.e. 262 L seeds/ha) rate of oat had greater levels of water tension (-29 and -60 kPa, respectively) than those seeded with the low (i.e. 131 seeds/ha) rate, which had an average water tension of -19 kPa. Oat plots seeded at the high rate, thus, had much drier soil than plots established using lower seeding rates in those years. Plots planted with the high seeding rate of rye also had greater water tension (i.e. drier soil) than those planted with the low seeding rate in 2003. Rainfall observations indicated that May was the driest month (i.e. 46 mm rainfall) in 2002, whereas rainfall amounts were much higher in May of 2003 and 2004 (i.e. 95 and 91 mm, respectively). In 2002, the highest monthly rainfall total for the entire three-year study (i.e. 132 mm) was received in June (Table 5).

Table 3. Post-application recovery of *Metarhizium anisopliae* CFUs from soil following at-plant granular (MaG) or post-emergence aqueous spray (MaS) formulations combined with cereal cover crops in sugarbeet, St. Thomas, ND, USA, 2002–2004.

	CFUs/g dry soil ± SD		
Year/treatment[a]	0 DAT[b]	30 DAT	60 DAT
2002			
Oat-low + MaG	247 ± 102	NT[c]	1902 ± 1956
Oat-low + MaS	8285 ± 4515	1527 ± 345	
Oat-high + MaG	530 ± 481	NT	905 ± 672
Oat-high + MaS	10867 ± 1663	1467 ± 1118	
Rye-low + MaG	467 ± 560	NT	1090 ± 1164
Rye-low + MaS	11247 ± 6764	872 ± 838	
Rye-high + MaG	622 ± 394	NT	1215 ± 873
Rye-high + MaS	16430 ± 11481	1527 ± 1151	
MaG	1185 ± 713	NT	1875 ± 1702
MaS	10310 ± 4466	1652 ± 1093	
2003			
Oat-low + MaG	62 ± 125	0	NT
Oat-low + MaS	1580 ± 747	372 ± 528	
Oat-high + MaG	125 ± 250	0	
Oat-high + MaS	4092 ± 3775	247 ± 339	
Rye-low + MaG	92 ± 119	0	
Rye-low + MaS	3182 ± 1599	155 ± 120	
Rye-high + MaG	60 ± 69	30 ± 60	
Rye-high + MaS	4530 ± 3456	560 ± 678	
MaG	62 ± 125	0	
MaS	2560 ± 331	217 ± 257	
2004			
Oat-low + MaG	122 ± 174	92 ± 185	NT
Oat-low + MaS	58800 ± 40984	4465 ± 1771	
Oat-high + MaG	122 ± 174	0	
Oat-high + MaS	46300 ± 9998	2277 ± 1888	
Rye-low + MaG	155 ± 237	62 ± 125	
Rye-low + MaS	55800 ± 17967	1655 ± 1647	
Rye-high + MaG	312 ± 473	0	
Rye-high + MaS	55750 ± 11800	2030 ± 385	
MaG	92 ± 185	437 ± 718	
MaS	49175 ± 15575	1777 ± 1402	

[a]Cover crops were sown at 0, 131 and 262 L seeds/ha in 2002 and 2003, and 0, 186 and 374 seeds/m^2 in 2004.
[b]DAT, Days after treatment.
[c]NT, Not tested.

4. Discussion

Overall, the results of this investigation indicated that conidia of *M. anisopliae* isolate ATCC 62176 are capable of surviving for up to 60 DAT under field conditions in a sugarbeet production system. Our general findings on persistence of the fungus in a row crop field environment are supported by those of Pilz, Enkerli, Wegensteiner, and Keller (2011), who observed survival of *M. anisopliae*, applied on a barley grain

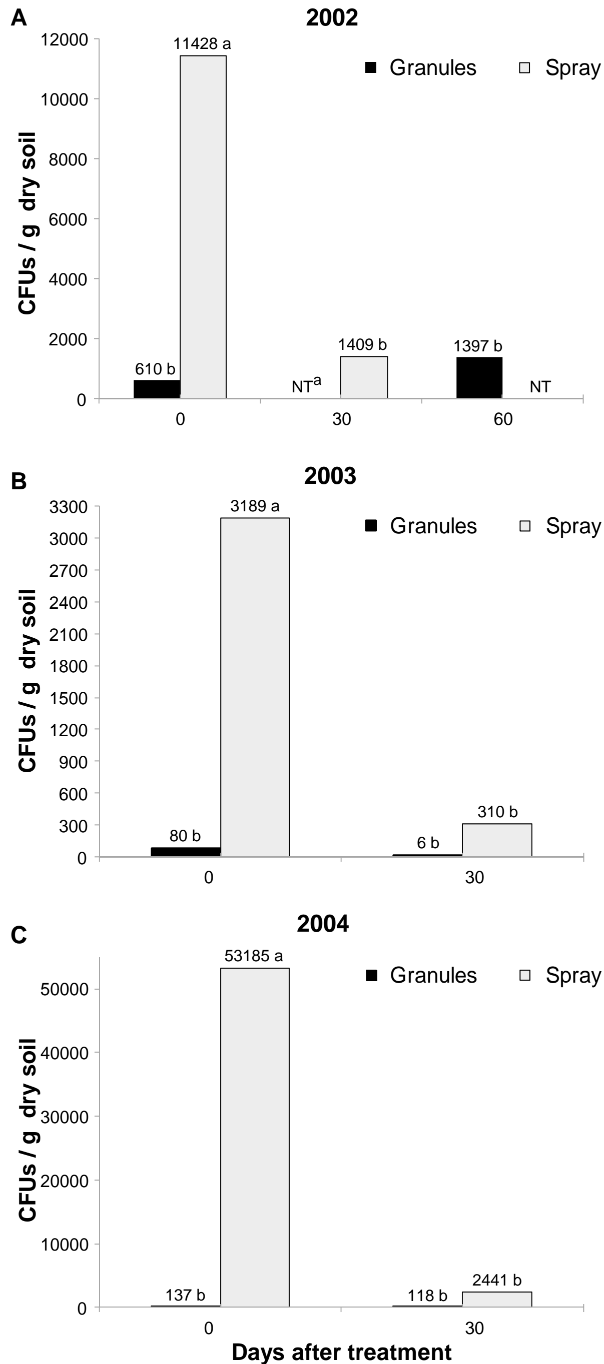

Figure 1. *Metarhizium anisopliae* CFUs per gram of dry soil at 0 and 30 or 60 DAT with granular and aqueous spray applications of the fungus at St. Thomas, ND, USA, 2002 (A), 2003 (B) and 2004 (C). Note: Means within a year followed by the same letter are not significantly different from each other (LSD, $\alpha = 0.05$).
[a]NT, Not tested.

Table 4. Average soil temperature and soil water potential after canopy formation (i.e. two-week average from 15 to 29 d after planting) of living oat, *Avena sativa* L., and rye, *Secale cereale* L., cover crops in sugarbeet, St. Thomas, ND, USA 2002–2004.

Cover type and seeding rate	2002		2003		2004	
	Soil temperature (°C)	Soil water tension (−kPa)	Soil temperature (°C)	Soil water tension (−kPa)	Soil temperature (°C)	Soil water tension (−kPa)
Oat, low rate[a]	23	19	17	19	16	31
Oat, high rate	23	60	17	29	24	19
Rye, low rate	23	18	15	12	20	31
Rye, high rate	23	21	NA[b]	28	16	28
No cover	21	NA[b]	15	17	16	NA[b]

[a]Cover crops were sown at 0, 131 and 262 L seeds/ha in 2002 and 2003, and 0, 186 and 374 seeds/m^2 in 2004.
[b]Data not available due to sensor malfunction.

substrate, for up to 15 months post-application in maize, *Zea mays* L., fields. In the present study, significant formulation × sampling date interactions occurred in all field trials, thus indicating that *M. anisopliae* formulation, delivery technique and application timing can affect the success of this fungus as a microbial tool for *T. myopaeformis* management. This finding is supported by Jaronski (2007) who suggested that conidia production and application methodology can impact persistence of entomopathogenic fungi. There was no concrete evidence to suggest increased persistence of *M. anisopliae* conidia in cover-cropped plots. However, oat cover crop plots generally had higher numbers (mean = 1045 CFUs/g soil) of viable conidia at 30 DAT than rye plots (mean = 689 CFUs/g soil). This also could have been a product of the markedly different canopy architecture produced by oat and rye plantings, although that was not a specific component of this study. It is important to note that these numbers reflect the results from relative sampling

Table 5. Post-application weather information, St. Thomas, ND, USA, 2002–2004.

Year	Month	Soil temperature (°C) at 10 cm depth	Solar radiation (MJ/m^2)	Rainfall (mm)
2002	May	8	22	46
	June	20	22	132
2003	May	13	18	95
	June	20	22	72
2004	May	12	17	91
	June	17	21	20

Source: NDAWN (2004).

methodology. As such, these results should not be interpreted as a granule- versus liquid-based formulation comparison. Markedly different placement methodology (i.e. in-furrow granules or surface-banded sprays over rows) was used to deliver the two fungus formulations, yet the same volume of soil was collected for persistence assessments. Previous investigators observed that conidia do not readily percolate downward into the soil profile, especially in silt loam and silty clay loam soils such as those in which we conducted our experiments (Ignoffo, Garcia, Hostetter, and Pinnell 1977; Storey and Gardner 1988). Thus, there would have been little to no vertical movement of spores from either the granules applied to the upper portion of the narrow seed furrow or the surface-applied liquid sprays. Therefore, the relative persistence and changes in CFUs per gram of soil over time are the most germane findings in these assessments.

It is possible that the rye cover crops established in this experiment could have imposed allelopathic impacts on *M. anisopliae* in the rhizosphere microenvironment. Several investigators have shown that roots of some plant species produce antimicrobial metabolites to inhibit plant pathogenic fungi as part of a defence system (Bais, Walker, Schweizer, and Vivanco 2002; Bais, Prithiviraj, Jha, Ausubel, and Vivanco 2005; Bais, Weir, Perry, Gilroy, and Vivanco 2006). Larkin, Griffin, and Honeycutt (2010) observed 12.5–19% reductions in the incidence of black scurf (causal organism: *Rhizoctonia solani* Kühn) in potato, *Solanum tuberosum* L., when a winter rye cover crop was established in different crop rotation schemes.

Less is known about allelochemical effects of plant-produced chemicals on entomopathogenic fungi. Lacey and Mercadier (1998) found that several plant-produced alkaloid compounds were capable of inhibiting germination of conidia of the entomopathogenic fungus, *Isaria fumosorosea* (Wize) Brown and Smith (formerly *Paecilomyces fumosoroseus*), by at least 50%. Similarly, Inyang, Butt, Doughty, Todd, and Archer (1999) observed that plant-produced isothiocyanates resulted in inhibition of germination and subsequent growth by *M. anisopliae*, as well as its ability to infect the mustard beetle, *Phaedon cochleariae* F. Although the findings of previous investigators suggest the possibility of allelopathic impacts on *M. anisopliae* in our study, further research would be required to confirm such an interaction and identify the specific mechanism for its effect.

Cover crops also appeared to impact soil moisture. Higher cover crop seeding rates tended to dry the soil more than low rates, which was probably a result of evapotranspiration. However, our results suggest the potential for resiliency of *M. anisopliae* conidia over time in the microhabitat provided by field conditions in the dryland (i.e. non-irrigated) sugarbeet production system within which we conducted our experiment. These findings correspond with the observations of previous authors who observed persistence of fungal entomopathogens for up to three years in agricultural soil habitats (Vänninen, Tyni-Juslin, and Hokkanen 2000; Vänninen 1995).

In our study, viable *M. anisopliae* conidia persisted in soil for at least four weeks after applications at temperatures ranging between 13 and 26°C and soil water tension values ranging from -19 to -60 kPa (Table 4). Our results relating to soil temperature impacts on survival of *M. anisopliae* conidia agree with previous research, which showed that temperatures between 15 and 30°C are suitable for growth of multiple isolates of *Beauveria bassiana* (Balsamo) Vuillemin and *M. anisopliae* (Jaronski 2007). Our findings also support those of Clerk and Madelin

(1965) who suggested a range of 8–25°C as being adequate for *M. anisopliae* conidia survival, and Ibrahim, Butt, and Jenkinson (2002) who suggested that low humidities (<90%) impede entomopathogenic fungus germination and sporulation. Our results also correspond with those of Walstad et al. (1970) who reported temperatures within a range of 15–35°C and humidity exceeding 92% to be optimal for *M. anisopliae*, as well as Daoust and Roberts (1983) who observed high survival of *M. anisopliae* conidia at the extremes of 0 and 100% humidity and reduced shelf life in storage at intermediate humidity levels.

The two *M. anisopliae* delivery methods used in our experiment resulted in interesting trends. Concentrations of viable conidia increased considerably in plots treated with fungus granules between 0 and 60 DAT. This suggests the likelihood that some degree of post-application sporulation and multiplication could have occurred on fungus granules in the soil microenvironment. This fungus has been observed to readily sporulate on corn grit granules placed on the surface of moist non-sterile soil (S.T. Jaronski, personal observations). Such increases would not likely contribute substantially to the total overall concentration of conidia in soil, but would certainly do so in the immediate zone around individual granules. Regardless, this finding demonstrates the possibility of conidial persistence for up to 60 DAT when the fungus is applied via modified in-furrow placement to soil as a planting-time granule. Survival and proliferation of conidia applied via the granular formulation which, albeit, only occurred during the first year of our study, could have been partially influenced by the nutritive properties of the granular carrier (i.e. corn grit substrate). Also, placement of fungus conidia in a manner that provided protection from the detrimental effects of solar radiation and high temperatures, such as the modified in-furrow placement of granules in this experiment, also could have prolonged the field efficacy of this biocontrol fungus.

The large numerical increases in CFUs per gram of sampled soil observed in our 2002 plots that received modified in-furrow applications of corn grit-based *M. anisopliae* granules could have, in part, been impacted by existing soil humidity and subsequent rainfall events. Moore, Langewald, and Obognon (1997) observed greater viability in conidia that were gradually hydrated by exposure to high relative humidity and then suspended in water when compared to conidia that were rapidly rewetted after deployment. Our 2002 plots were established in moderately moist soil in May, and relatively low rainfall amounts (i.e. 46 mm) were received that month. Subsequently, much higher rainfall events occurred during June (i.e. monthly total of 132 mm) of that year. Our observation of consistently large increases in CFUs per gram of soil in *M. anisopliae* granule-treated plots across treatments in 2002, irrespective of whether a cover crop was used, would, therefore, support the findings of Moore et al. (1997) and those of Jaronski (2007) who observed favourable levels of conidiation in mycelial granules of both *M. anisopliae* and *B. bassiana* following rehydration in non-sterile field soil. Results of the present study also reflect those of Jaronski and Jackson (2008) in which efficacy of *M. anisopliae*-based (i.e. isolate F52) microsclerotial and corn grit granules against *T. myopaeformis* larvae was greatest under higher levels of available soil water. In that study, efficacy of the corn grit granular formulation of *M. anisopliae* was greatly reduced under drier soil conditions. However, as pointed out by Jaronski (2007), overgeneralisation and application of such phenomena should be avoided, as it does not universally occur

across *M. anisopliae* isolates (Ekesi, Maniania, and Lux 2003) or other genera such as *Beauveria* and *Isaria* (S.T. Jaronski unpublished).

Soil moisture has been previously identified as crucial for germination of fungus spores in the soil microenvironment (Walstad et al. 1970). Within this context, *M. anisopliae* granules could act in a manner similar to that of conventional at-plant chemical insecticides that typically involve a latency period before the toxicant is activated (i.e. eluted from the granular carrier) by rainfall and/or soil moisture. Delivering fungus conidia to the target zone via a corn grit-based granular carrier would likely also involve a pre-patent period after application during which time the nutritive carrier granules become hydrated. Conidia then germinate and grow into mycelia in and on the granules. The fungus subsequently conidiates to produce a high number of fresh conidia that are loosely bound on the granule surface and in the soil immediately adjacent to the granule. Fungi would likely have a slightly longer pre-patency period than conventional chemical insecticides; however, delayed and extended activity of a fungal entomopathogen could be beneficial in managing target insects such as *T. myopaeformis* larvae that feed on sugarbeet roots up to two months after the application of at-plant granular formulations. The use of a nutritive granular carrier also offers the advantage of fungal regrowth and conidiation, which can lead to multiplication of the originally deployed concentration of fungus.

Survival of *M. anisopliae* conidia in plots treated with the spray formulation dropped dramatically between 0 and 30 DAT. Losses of viable conidia in spray plots averaged 91% during that 30-d interval in this study. Low survival rates of conidia applied via post-emergence aqueous sprays banded on the soil surface could have perhaps resulted from exposure to more solar radiation than those applied to the protective seed furrow as granular formulations. This finding is supported by results of previous research that indicated a half-life of 3–4 h for fungal conidia applied in the field and subjected to natural sunlight conditions (Roberts and Campbell 1977; Braga et al. 2001a). Similarly, a half-life of about one day was reported by Inglis et al. (1997) who applied conidia in a North American shortgrass prairie habitat. Conidia applied via aqueous spray applications in our study also could have been negatively impacted by exposure to high temperatures on the soil surface. Optimal temperatures for survival, development and pathogenicity by entomopathogenic fungi are believed to be in the range of 20–30°C (McCoy, Samson, and Boucias 1988). Although soil temperatures experienced by granular formulations applied into and adjacent to the seed furrow in May of our study were within this range, the post-emergence spray-applied treatments would have been exposed to much higher temperatures on the soil surface during the month of June. Soil surface temperatures of 48–50°C have been observed in open, bare-ground microhabitats near mid-season sugarbeet plants at the time post-emergence liquid biopesticides would be applied for *T. myopaeformis* management in sugarbeet (S.T. Jaronski unpublished data). Fungus survival under these conditions also could be compromised because high surface temperatures tend to accelerate desiccation of the upper (i.e. 0.5- to 1-cm) portion of the soil profile.

Fungus formulation and possibly strain (e.g. drought- heat- or solar radiation-tolerant) are likely to play major roles in the commercial feasibility of *M. anisopliae* or other candidate entomopathogenic fungi for *T. myopaeformis* control under such variable environmental conditions as those common to dryland sugarbeet production in north central North America. Synchronising infectivity of the fungus with the presence of a susceptible stage of the target pest will also be an important factor for

increasing the likelihood of successful pest management via fungus-based biopesticides such as the *M. anisopliae* granular and spray formulations examined in this study.

Our findings suggest that, although formulation had a major impact on persistence of *M. anisopliae* isolate ATCC 62176, favourable numbers of viable conidia were present in the field from 30 to 60 DAT (i.e. during peak adult activity and larval feeding periods of *T. myopaeformis*) in both spray- and granule-treated plots. No significant benefits were observed for either oat or rye cover crops in relation to conidia persistence. However, additional research is needed to ascertain whether subterranean or soil surface microhabitats provided by cereal cover crops impact the efficacy of this fungus against *T. myopaeformis* in sugarbeet. Dregseth et al. (2003) demonstrated that integrating oat cover crops with a conventional organophosphate insecticide (e.g. terbufos) tended to increase levels of *T. myopaeformis* control when compared to insecticide-only plots; however, the specific mechanism for increased efficacy provided by the cover crop was not identified. Shapiro-Ilan, Gardner, Wells, and Wood (2012) observed enhanced persistence and efficacy of endemic *Beauveria bassiana* (Balsamo) Vuillemin for pecan weevil, *Curculio caryae* (Horn), control by using a white clover, *Trifolium repens* L., cover crop in pecan, *Carya illinoinensis* (Wangenheim) K. Koch, orchards; however, those authors also could not determine the factors responsible for the increases. Therefore, additional research is needed to further understand the biotic and abiotic interactions of entomopathogenic fungi with different plant habitats and under a variety of cover crop environments. Such knowledge could lead to further enhancements and uses for these important organisms in bio-based pest management. Future research also should seek to determine if production systems that combine *M. anisopliae* with cereal cover crops can provide an effective integrated strategy for management of *T. myopaeformis* in sugarbeet.

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