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INTEGRATION OF A RESISTANT SUGARBEET VARIETY WITH THE INSECT PATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* TO MANAGE THE SUGARBEET ROOT MAGGOT.

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INTRODUCTION

The insect pathogenic fungus, *Metarhizium anisopliae*, has been under study for the control of *Tetanops myopaeformis*, the sugarbeet root maggot (SBRM), for several years. It has not been very effective by itself in the face of the heavy insect pressure that routinely occurs in the northern Red River Valley, esp. in the St. Thomas ND vicinity. Integration of a microbial biocontrol agent with other pest management tools may provide economic management of pest damage. Use of *M. anisopliae* with a cover crop is a strategy under recent assessment (Majumdar et al., 2004).

Another approach may be to couple an SBRM resistant sugarbeet with the fungus. Campbell et al. (2000a) developed and released two breeding lines, F1015 and F1016, with resistance or at least tolerance to SBRM feeding. One disadvantage, however, is that these lines currently have commercially inferior yields. Nevertheless, these lines offer an opportunity to test whether coupling plant-resistance with an otherwise insufficiently effective microbial might significantly improve control and yield. To our knowledge, this approach has not been tested in any other crop. We conducted small plot field trials in 2003 and 2004 in which a resistant variety, F1015, and a SBRM susceptible variety, Beta 3820, were combined with applications of *M. anisopliae*. Our goal was to determine whether the fungus would reduce damage (increase yield) to a significantly greater extent in a resistant variety than in a susceptible variety.

MATERIALS AND METHODS

We hypothesized that root damage reduction and yield increase would be significantly greater when the *Metarhizium* was coupled with a resistant variety than when it was paired with a susceptible sugar beet. The experiment examined four treatments: an SBRM-susceptible variety, Beta 3820 (Beta Seed Inc.), with and without *Metarhizium*, and a resistant variety, F1015, with or without *Metarhizium*. The trials were arranged in a randomized complete block design with four replicates. Each plot consisted of 4 rows, 11 m (36 ft) long, separated by four rows of a susceptible commercial hybrid (Hilleshog Resist™) Each replicate block was separated from the others by a 7.3 m (24 ft) bare ground buffer zone.

In both years, the trials were conducted at Buzz Baldwin Farms, St. Thomas ND. Seed was planted 2.5 cm deep in rows spaced 56 cm apart. All plots were thinned to approximately 76,500 seedlings per hectare (31,000 plants/acre) before colonization of the field by adult flies. Weeds were controlled with micro-rate applications of herbicides, cultivation, and hand weeding. Fungicides for Cercospora leaf spot control were applied when conditions were favorable for disease development.

The fungus, *M. anisopliae* strain MA1200 (ATCC62176), was applied twice during the crop season, first as a granular formulation at planting, and later as an aqueous, post-emergent spray. The fungus was produced in a biphasic liquid-solid substrate production system at Sidney MT. Conidia were harvested from the solid substrate with an air classification system. Granules were composed of conidia bound to 16-20 mesh corn grits (Snack Grits® 114RB, Conagra Foods Corp.) with 20% polyoxyethylene sorbitan monolaurate (Tween 20®) to a target of $7x10^{10}$ conidia/Kg ($5x10^4$ conidia per granule). Granules were applied at 28Kg/ha (25 lb/Ac), band over row, with a bicycle applicator and lightly incorporated. A second application of fungus was made just before predicted peak fly oviposition. This application was an aqueous spray of conidia applied in a 10-12 cm (4-5 in) band over row directed to the base of each seedling. Rate of *Metarhizium* was $1.3x10^{11}$ conidia/L (equal to $5x10^{13}$ conidia/ha). Total spray volume was 374 L/ha (40 gal/Ac), a volume we thought sufficient to carry spores into the top several cm of soil.

Root damage ratings were performed in August of each year. Ten beets from the outer two rows of each plot were rated for damage on the 0-9 scale of Campbell et al. (2000) (0 = no damage; 9 = >75% of root surface with feeding scars). Yield determinations were based on roots harvested from the two center rows of each plot in September. Sugar and impurity analyses were conducted by the American Crystal Sugar Company Tare Laboratory (East Grand Forks, MN).

Soils were sampled immediately after application of *Metarhizium* spray, and 14 and 28 days post application, to determine *Metarhizium* titers and conduct bioassays. Approximately 25 cm^2 of soil in the sprayed swath in each of four locations were sampled in each plot; all samples from a plot were combined. Separate samples were taken from the top 1-2 cm and the next 1-2 cm of the soil surface. These soil samples were chilled and transported to Sidney

MT for serial soil dilution plating. At Sidney samples were mixed thoroughly and two separate 10-gram samples were then suspended in 90 ml aliquots of 0.1% polyoxyethylene 20 sorbitan monooleate (Tween 80®). Suspensions were mechanically agitated 2 minutes then sonicated 5 minutes. Two additional 10-fold dilutions were subsequently made from the initial suspension. All three dilutions were used to inoculate four plates of a selective agar medium (modified from Chase et al., 1986) for each dilution. The medium consisted of Gerber Oatmeal Cereal for Baby® (20g/L), agar (20 g/L), dodine as Syllit® 65W (0.46 g/L), and gentomycin (Gentocin ®) (1 ml/L). The inoculated Petri plates were incubated 5-7 days, until *Metarhizium* colonies were discernible, and the number of colonies were then counted. The dilutions yielding 20-100 colonies per plate were used to calculate the Colony Forming Units (CFU) per gram of dry soil. These CFU are assumed to represent conidia and/or blastospores rather than hyphal fragments (Warcup, 1955).

The remaining soils were refrigerated until August of each year for bioassays with diapausing, third instar root maggot larvae collected that same year. Only the surface (top 1-2 cm) soil samples were bioassayed. For bioassays, 60-gram samples were drawn from each sample bag; rehydrated to a calculated 15% field saturation, based on predetermined moistures and field saturation levels; and then apportioned equally to three 60 ml plastic condiment cups. Ten larvae were then added to each cup. Cups were sealed and incubated at 24° C. for three weeks at which time mortality and prevalence of mycosis were determined.

Temperature and moisture of the upper layers of soil, where the primary interaction between neonate SBRM larva and *Metarhizium* is thought to occur, were monitored. Temperature probes were positioned in a row at 2 and 4 cm depths. Soil moisture probes (ECH2O ® probes, Decagon Inc.) were placed at 2-4 and 6-8 cm depths. These probes, which are flat, 25 cm long by 3 cm wide blades, were placed horizontally in the soil but tilted along their long axes to prevent pooling of water on the sensor surface. All probes were connected to data loggers for hourly measurements during the entire field season.

RESULTS AND DISCUSSION

In 2003 maggot pressure as measured by root damage was moderate (mean rating of 5.8 in the susceptible Beta 3820) (Table 1). There were no significant differences due to *Metarhizium* applications in root damage or yield, within variety, even though there were numerical increases in all categories (except F1015 percent sugar). Differences in root damage and yield were significant between the two varieties.

In 2004 maggot pressure was severe (mean damage rating of 6.8 in the susceptible Beta 3820) (Table 1). Use of at-planting and "peak-fly" spray applications of *Metarhizium* did not significantly affect root damage, or yield (weight of beet per acre, percent sugar, or extractable sugar per acre) in either sugarbeet variety (Table 1). However, there were small numerical increases in yield components for F1015 plus *Metarhizium*, versus decreases with Beta 3820 plus *Metarhizium*, however.

Table 1. Summary of 2003 and 2004 field trials in which *Metarhizium anisopliae* Strain MA1200 was paired with a sugarbeet root maggot susceptible beet variety (Beta 3820) and with a resistant variety (F1015). Means in any column followed by the same letter are not significantly different (LSD test, p=.05).

			Yield		
Year	Treatment	Root Damage[1] (± S.D.)	Tons per Acre (± S.D.)	Percent Sugar (± S.D.)	Lb. Extractable Sugar per Acre (± S.D.)
2003	Beta 3820	5.8 (0.18) a	23.9 (4.14) a	17.3 (0) a	7596 (1313) ab
	Beta 3820 + *Metarhizium*	5.3 (0.31) a	25.5 (5.26) a	17.9 (0.54) a	8439 (1561) a
	F1015	4.0 (0.28) b	21.5 (1.27) a	16.2 (0.33) b	6270 (512) b
	F1015 + *Metarhizium*	3.6 (0.06) b	23.2 (2.25) a	16.0 (0.15) b	6659 (633) ab
	LSD Value (p = .05)	1.10	4.65	0.54	1407.5
2004	Beta 3820	6.8 (0.18) a	14.6 (0.52) b	14.6 (0.9) ab	4482 (454) a
	Beta 3820 + *Metarhizium*	6.7 (0.16) a	15.3 (0.64) ab	15.0 (0.9) a	4360 (337) a
	F1015	3.4 (0.23) b	16.4 (1.31) ab	13.5 (0.1) c	4434 (319) a
	F1015 + *Metarhizium*	3.2 (0.18) b	16.7 (1.09) a	13.8 (0.6) bc	4598 (484) a
	LSD Value (p= .05)	0.80	2.11	1.08	623.7

1 Root Damage is on 0-9 scale.

If there were to be an enhancement of *Metarhizium* efficacy by coupling with a resistant hybrid, then increase in yield components would be significantly greater with F1015 (the resistant variety) than with Beta 3820. Statistically significant enhancement of *Metarhizium* efficacy by combination with a resistant hybrid did not occur in either year even though there were numerical increases in tons of beets and extractable sugar per acre (Table 2).

Table 2. Changes in sugarbeet yield components associated with SBRM management by *Metarhizium* in a susceptible and resistant beet variety. In each case yield from the variety alone was subtracted from yield of variety plus *Metarhizium*; Percent change is that difference divided by yield from variety alone.

Year	Variety	Change in ton/acre	% Change	Change in percent sugar	% Change	Change in lb. sugar per acre	% Change
2003	Beta 3820	+1.57	7%	+0.6%	4%	+843	11.1%
	F1015	+1.68	8%	-0.2%	-1%	+389	6.2%
		ns		ns		ns	
2004	Beta 3820	+0.72	5%	-0.3%	-2%	+116	3%
	F1015	+0.29	2%	+0.2%	2%	+154	3%
		ns		ns		ns	

In both years the untreated F1015 suffered significantly less root damage (69% and 50% of the susceptible Beta 3820 in 2003 and 2004, resp., Table 1). In 2003 under moderate insect pressure, root yield of F1015 was nominally 91% of Beta 3820 (and not significantly different), but in 2004, under heavy insect pressure, yield from F1015 was numerically better (107%) than Beta 3820. Sugar content, however, was consistently lower with F1015 in both years. Consequently, extractable sugar per acre was not significantly different between the two hybrids.

Some insight into these observations is provided by the bioassays of field soils and determination of fungus titers in the top layer of soil where we believe neonate SBRM larvae interact with *Metarhizium* conidia.

Table 3. *Metarhizium* titers in 0-2 cm and 2-4 cm layers of soil in the sprayed swaths of treated plots, immediately after application, 14 and 28 days post-application. In 2004 the 14-day samples were not collected.

			Mean (±S.D.) Colony Forming Units per gram dry soil		
	Treatment	depth	0	14	28
2003	B3820	0-2 cm	2366 (2211)	1844 (1086)	625 (234)
		2-4 cm	5975 (5517)	15716 (9981)	11416 (13737)
	B3820+*Metarhizium*	0-2 cm	283438 (167319)	40909 (20164)	18497 (13680)
		2-4 cm	13619 (7524)	18025 (8597)	14322 (8110)
	F1015	0-2 cm	3366 (4209)	7297 (6995)	3025 (4012)
		2-4 cm	2725 (4599)	3288 (3611)	2663 (4276)
	F1015+*Metarhizium*	0-2 cm	209625 (113354)	54163 (35210)	41584 (15716)
		2-4 cm	11331 (13341)	13984 (11780)	14425 (16721)
2004	Beta 3820	0-2 cm	0	nd	0
		2-4 cm	0	nd	0
	B3820 + *Metarhizium*	0-2 cm	128063 (69438)	nd	4431 (2635)
		2-4 cm	11828 (4006)	nd	nd
	F1015	0-2 cm	0	nd	0
		2-4 cm	0	nd	0
	F1015 + *Metarhizium*	0-2 cm	168125 (61759)	nd	16694 (10850)
		2-4 cm	6909 (3906)	nd	nd

It was not possible to accurately determine fungus titers following application of *Metarhizium* granules in furrow. One would have to sample a fixed volume of soil immediately surrounding the granules to obtain an accurate, realistic estimate of infectious titers. Past work (Jaronski, unpublished data), revealed that excessive variability makes such observations almost meaningless. Therefore, such sampling was not performed. Greater accuracy was possible in sampling soils following band-over-row spray applications of fungus spores.

In both years the initial *Metarhizium* titers in the top 2 cm of the soil profile were 11-24 times higher than in the next 2 cm (Table 3). In 2003 these differences decreased during the subsequent 28 days, by which time CFU levels were similar in the two profiles (Table 3). Several problems prevented us from determining detailed persistence of fungus in the 2004 field trial. Background levels in 2003 untreated plots were low but unexpectedly greater than past experience--and 2004 data--would predict.

Laboratory observations of soil percolation by *Beauveria* and *Metarhizium* conidia into clay and silty clay soils revealed that spores are generally retained in the top 1-2 cm of the soil profile (Jaronski, unpublished). Similarly, Storey et al. (1989) reported that most *B. bassiana* conidia, which are much smaller and rounder than *M. anisopliae*, remained in the upper 5 cm (their smallest depth increment) of the profiles of the Cecil sandy clay loam soil following application to the soil surface. Such data influenced our sampling scheme. Nevertheless, there was substantial source of bias in sampling soils, bias connoted by the large standard deviations of the CFU determinations. The variability observed was mostly explained by plot-to-plot variability rather than between sub sample variability, which was generally very small.

The CFU data indicate a rapid decrease in titers to levels that would be marginally efficacious, at least for third instar larvae. Bioassays of MA1200 in several clay and silt clay soils using diapausing third instar larvae indicate an LC_{50} for that stage of ca. $2.5x10^5$ conidia/gram soil at 15% and 30% field saturation, and a much higher LC_{50} at 10% saturation (Jaronski, unpublished data). The LC_{50} for younger larvae, esp. first instars, would presumably be lower, but such data have not yet been generated in soil bioassays because of very poor control larval survival. We observed initial titers of $1.3\text{-}2.8x10^5$ CFU (spores)/gram soil, but our estimates reflect uniform distribution of spores throughout the top 2 cm. It is likely that the spores could have been more concentrated within the top 5 mm of the soil profile. Fungal persistence observed in 2003 and 2004 was much shorter than observed persistence in 2001-2002 or in the Sidney MT field trials (2001-2004) (Jaronski, unpublished data).

Table 4. Mortality of diapausing third instar SBRM larvae exposed to field soil samples taken 0, 14, and 28 days after application of the *Metarhizium* sprays. Data are means of three replicate bioassays.

		Mean mortality of SBRM larvae (± S.D.)		
		Days post-application		
	Treatment	0	14	28
2003	Untreated B3820	5% (3.4%)	0%	0%
	B3820 + *Metarhizium*	13% (0.7%)	10% (0%)	7% (5.5%)
	Untreated F1015	0%	0%	5% (3.4%)
	F1015 + *Metarhizium*	17% (1.7%)	7% (5.5%)	10% (0%)
2004	Untreated B3820	5% (3.4%)	0%	0%
	B3820 + *Metarhizium*	100% (0%)	96% (10.8%)	80% (10.8%)
	Untreated F1015	2% (7%)	0%	0%
	F1015 + *Metarhizium*	99% (3.4%)	80% (0%)	96% (0%)

Bioassays of field soils (Table 4) did not correlate well with the CFU determinations or with laboratory bioassays conducted in the past several years. Whereas *Metarhizium* titers were on the order of $2\text{-}3x10^5$ CFU/g soil in the top 2 cm of the sprayed swath in 2003, mortality of SBRM larvae in bioassays with these soils was only 13% and 17%, much lower than expected from LC_{50} estimates from laboratory bioassays. In 2004, however, initial CFU levels were lower than in 2003 -- $1.2\text{-}1.6x10^5$ CFU/g -- yet larval mortalities (and prevalence of mycosis) were very high (99-100%), much higher than in the previous year and also higher than expected from previous multiple-dose laboratory bioassays. And, as titers dropped even further, bioassay mortality from *Metarhizium* remained very high. Reduction in root damage was minimal in that year. We cannot explain these discrepancies. *Metarhizium* is not known to multiply in non-sterile soils (McCoy et al., 2002) so soil bioassays should not have been biased in that regard.

In 2003 soil temperatures at the 2 cm level ranged from 13-32° C. during the 28 days subsequent to application of the *Metarhizium* sprays (Table 5). In 2004 soil temperatures were similar but briefly reached 40° C. Except for the 2004 maximum these temperatures are within the range for conidial persistence, germination, and fungal growth. Brief periods of 35-40° C. are not harmful to the fungus (Jaronski unpublished data). In 2003 moistures in the top 2-4 cm of soil were relatively constant, ranging between 21 and 26 m^3/m^3, levels permissive for *Metarhizium* persistence based on our experience. In 2004 the top layer was subject to periods of severe desiccation below Permanent Wilting Point on several occasions. These conditions may have been responsible for the more severe die-off of *Metarhizium* that year (Table 3).

The combination of *Metarhizium* with a resistant hybrid did not protect sugarbeet to any significant extent in our trials. While the supplementary data are conflicting, we believe the fungus was not sufficiently efficacious to properly test our hypothesis. Subsequent to these studies we have adopted a more virulent isolate of *M. anisopliae*,

Strain F52 (Earth Biosciences), and are developing an improved fungal granule. With these changes, efficacy of the fungus may be improved to the point where significant plant protection can be obtained. In addition, the combination of fungus and a resistant hybrid may be successful in the face of light to moderate maggot pressure (3-5 root damage rating in a susceptible hybrid).

Table 5. Summary of soil temperatures and moistures for first 28 days following application of *Metarhizium* sprays to plots. Water activities (Aw) were calculated from calibrations of Decagon ECH2O® probes of known gravimetric water contents and water activities of soil from the plots under observation.

	Temperature (°C.)		Moisture in 2-4 cm soil profile		Moisture in 6-8 cm soil profile	
	2 cm	4 cm	Moisture (m^3/m^3)	Water Activity (Aw)	Moisture (m^3/m^3)	Water Activity (Aw)
2003						
Mean	20.2	21.1	22.3	0.990	19.3	0.988
Min.	12.9	13.3	20.7	0.993	14.5	0.956
Max.	32.3	30.7	25.7	0.986	26.3	0.992
2004						
Mean	21.7	20.3	14.5	0.967	nd	-
Min.	7.8	10.2	5.6	0.769	nd	-
Max.	39.7	31.9	27.9	0.995	nd	-

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