Early application of *Metarhizium brunneum* for management of wireworm in popcorn, *Zea mays* var. *everta*.

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Abstract

Wireworm, the larval stage of the click beetle, is a family of subterranean, polyphagous plant pests, several species have been introduced to Canada from Eurasia. *Agriotes obscurus* L*,* and *A. lineatus*, L. can cause particular damage to a wide range of crops, including corn, *Zea mays*. *Metarhizium brunneum* (Hypocreales: Clavicipitaceae), is a fungus that parasitizes invasive wireworms and adult click beetles in the Fraser Valley. Unlike synthetic pesticides, it takes time to take effect. This study compared early and late applications of granulated *M. brunneum* conidia as a biocontrol for wireworm in popcorn (*Z. mays var. everta*). Using a randomized complete block design, we tested four treatments which were untreated control, oats and *M. brunneum* applied 24 days before seeding, oats applied 24 days before seeding, or oats and *M. brunneum* applied at seeding. We found no significant effect given the design used in this particular experiment; even though corn cultivation has not been possible in the field in question given the level of wireworm pressure prior to *M. brunneum* treatments being applied in previous years.

Key terms: entomopathogenic fungi, biocontrol, wireworm

Introduction

Wireworms are the polyphagous, root feeding, subterranean larval stage of adult click beetles. The species that are currently of greatest concern to farmers in British Columbia are *A. lineatus* L. and *A. obscurus*, L. with the latter being the most significant (Van Herk et al 2018). Both these species can live for up to 5 years as larvae in farm soils, while feeding on agricultural crops with a preference for Poaceous species, such as grass-based cover crops (Jansson et al 1991), and cereals like barley, rye, wheat, and corn (Andrews et al 2008). They can also burrow into roots and tubers, making crops like potatoes unmarketable (Andrews et al 2008). After several instar phases, wireworms pupate from spring to summer, emerging as adult beetles, which remain in their pupal burrows over winter, and come to the soil surface to reproduce the follow spring. Both wireworm species are alien and invasive species and were accidentally introduced from Eurasia (Andrews 2008). These wireworm species feed on living plants, including any plant material that touches the soil where they dwell. Wireworms have heightened feeding activity in spring and fall when they move laterally and come closer to the soil surface to feed (Kabaluk 2020). These periods are characterized by ideal soil moisture and temperature (11-18^{o}C) levels for wireworm activity.

Wireworm is a serious pest of crops in British Columbia. For example, the Delta and Surrey areas alone suffered $500,000 to $800,000 in crop losses caused by wireworms in 1994 (Berube 2007). It is also important to note that wireworms in general are attracted to CO_2 sources (Kabaluk 2020).

Organochlorine, organophosphate, and neonicotinoid insecticides have all been registered for wireworm control on Canadian farms. Previously permitted organochlorines included aldrin, dieldrin and heptachlor, which were all banned in the 1960s and 70s (Pest 2019) due to environmental persistence and negative effects on birds that consumed insecticidal granules (Bérubé 2007). Previously allowed organophosphates included phorate, chlorpyrifos and pyrethroid (bifenthrin), which were banned due

to deleterious effects on wildlife, especially birds (Pest 2015). Previously permitted neonicotinoids include thiamethoxam, clothianidin and bifenthrin. Two other neonicotinoids, thiamethoxam and clothianidin, are still allowed for limited use in Canada. Their registration as a permitted substance on farms is being reviewed due to negative impacts on pollinators, including honeybees (Canada 2019). If these remaining neonicotinoids are disallowed for use, no synthetic insecticides will be available for wireworm control in Canada (Pest 2015).

M. brunneum is an entomopathogenic member of the Clavicipitaceae family and is therefore closely related to the cereal endophyte ergot, *Claviceps purpurea* (Fr.) Tul., the ryegrass endophyte *Epichloë* spp. (Fr.) Tul. & C. Tul., and *Periglandula ipomoeae* U. Steiner, E. Leistner & Schardl., an endophyte of Convolvulaceae. All of these endophytes produce indole alkaloids that are toxic to potential herbivores and therefore of agricultural interest (Florea 2017). *M. brunneum* is an entomopathogen of a number of different arthropod species, but the strain selected for this study, LRC112, has shown a high degree of virulence in the wireworms of *Agriotes* spp. (Kabaluk 2014). This strain was isolated from a wireworm cadaver found near Agassiz, BC by Todd Kabaluk of Agriculture and Agri-Food Canada (Ericsson et al 2007). Currently, this fungus is one of the most promising wireworm biocontrols being developed in our region as an alternative to synthetic insecticides. *M. brunneum* has been observed to grow endophytically with corn (Kabaluk, pers. comm.).

Lab-based bioassays have found that wireworm mortality occurs within 1 to 4 weeks of *M. brunneum* application (Kabaluck 2014). Similar results may occur in the field.

As a pathogenic organism, conidia of *M. brunneum* require time, certain temperatures, moisture levels, and availability of appropriate substrate or host to commence growth. To achieve full pathogenicity, the organism also requires time to grow within the host wireworm. Infected wireworms may continue to feed on plant roots until death (Kabaluk et al 2005).

Soil temperature influences the time required for *M. brunneum* to infect and kill wireworms (Kabaluk et al 2005, 2020). *M. brunneum* growth increases with temperature between 12 and 30°C, then declines at temperatures above 30°C (Kabaluk et al 2005). Soil temperatures above 12°C are first observed in late spring in the Fraser Valley (Fig. 1). Wireworms are also most active in spring (April through June), when corn is typically planted (Andrews 2008). Newly germinated and emerging corn plants tend to be most vulnerable to wireworm damage.

M. brunneum mediated mortality of wireworm occurred more quickly at 18°C than at 12°C, and was not observed at 6°C (Kabaluk et al 2005). *M. brunneum* colony growth *in vitro* peaked at 30°C (Kabaluk et al 2005).

Wireworms avoid regions of soil with higher *M. brunneum* conidia concentrations (Kabaluk et al. 2005). *M. brunneum* applied between rows at planting may cause infected wireworms to move into crop rows, increasing damage.

Cleary the time of application prior to the seeding of corn may have an impact of *M. brunneum's* efficacy as a biocontrol for wireworms when applied in-situ for crop protection from certain pests. For the current study, the hypothesis being tested was whether *M. brunneum* (LRC112) would be more effective as a biocontrol for wireworms if applie d four weeks prior to seeding rather than at the time of seeding.

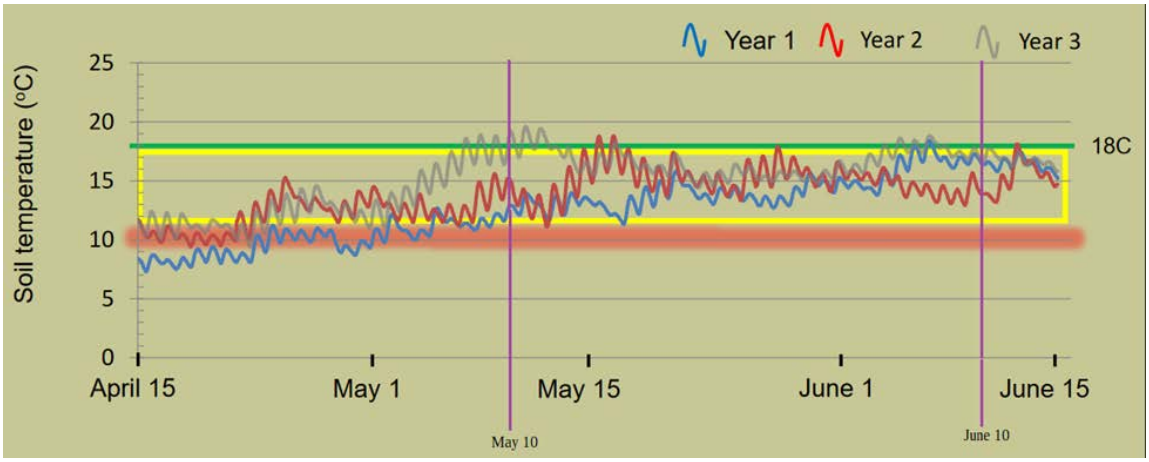

Figure 1. Soil temperatures (°C) at 20 cm April 15 –June 15 in Agassiz, British Columbia (Kabaluk 2007, 2020). Corn is typically planted between May 10 and June 10 (vertical lines) in the region.

Methods

The study was conducted on certified organic land at the KPU Orchard at the south end of Gilbert Rd. in Richmond, BC (Figure 2). Soil is shallow muck with 11% organic matter, over a Ladner silt loam. The experiment employed a randomized complete block design with eight replicates arranged pairwise from north to south (Fig. 3). This arrangement allowed the design to block against two directions of variation: the field to the north, and the field and fruit trees to the east, which were both source of migrating wireworms.

Within this experiment, the independent variable was corn kernel weights, and the dependent variable was the application of *M. brunneum* at four weeks prior to seeding and at the time of seeding.

The treatments were:

a) *M. brunneum* and oats applied four weeks prior to seeding
b) Oats applied four weeks prior to seeding
c) *M. brunneum* and oats applied at the time of seeding
d) Control (no treatment)

Figure 2. Aerial view of experimental site (orange rectangle) at the KPU Orchard, east of the Gilbert Road Community Garden, near the corner of Gilbert Road and Dyke Road in Richmond, BC.

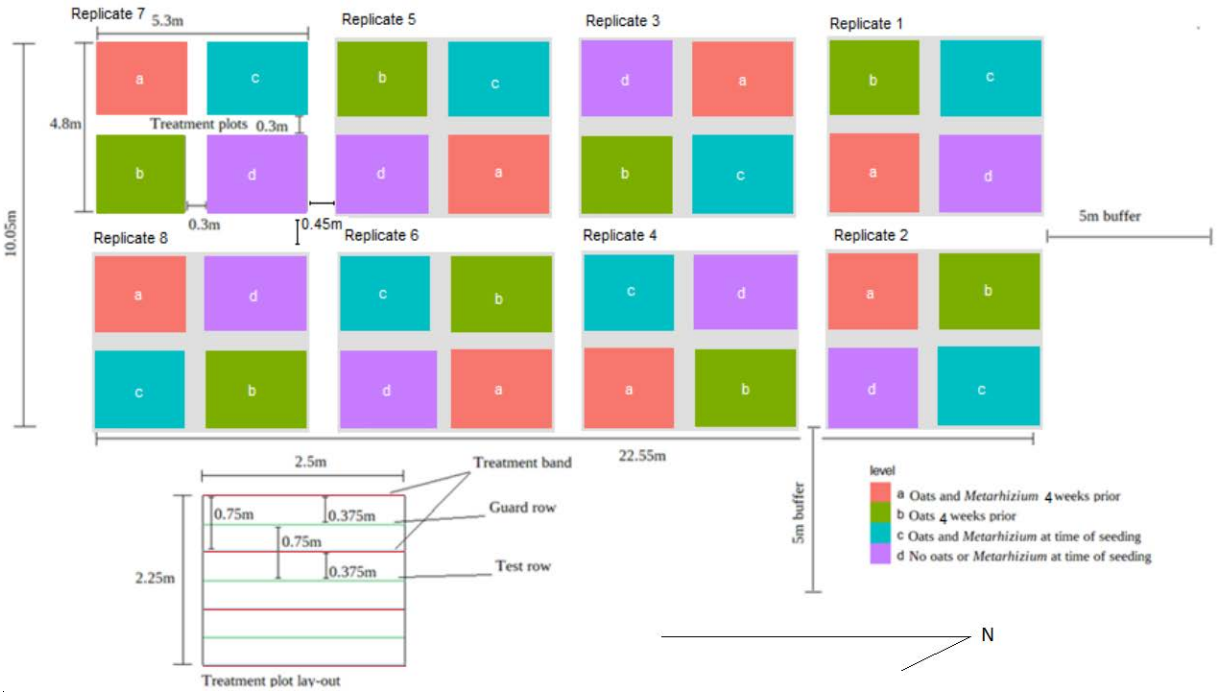

Figure 3: Experimental design, with plot and buffer dimensions and crop layout randomization (not to scale).

A cover crop of winter rye was incorporated at the experimental site with a roto-tiller on 17 April. Furlan et al (2020) found that freshly tilled meadow and pasture can distract wireworms from damaging emerging corn. Although, cover crop incorporation shortly before planting corn could have a similar effect, cover crop incorporation at the experimental site occurred more than a month before anticipated corn planting, so effects were most likely negligible.

M. brunneum and oats were applied to pre-treated plots on 10-11 June at a depth of 15 cm. All plots were cultivated with a power harrow attached to a walk-behind tractor (BCS, Milan, Italy) to a depth of about 7 cm on 1 July. Remaining treatments were applied and corn was direct-seeded at a depth of 6 cm and 15 cm in-row spacing on 4 July.

Figure 4. Treatment plot prior to final treatments being applied.

Figure 5. All treatment plots prior to final treatments being applied.

Figure 6. A treatment band with both treatments (*M. brunneum* and oats) applied.

Data collection

A soil core was collected from the treatment band in the northwest corner of each plot on 16 July. Soil samples were placed in brown paper bags marked by plot until 17 July, when they were sifted using a mesh lined plastic crate in half-oil-drum containers to enable wireworm collection.

After sifting, all collected wireworms were immediately placed in petri-dishes containing 5 x 5 cm pieces of moist paper towel. Dishes were wrapped in newspaper for transport then kept at room temperature. A carrot slice was placed in each dish for the wireworms to eat.

Wireworms were counted in core samples collected from the southwest corner of each treatment plots, and taken from within the treatment bands, at the time of seeding.

Crop emergence counts were conducted on 24 July. Plant height was measured on 10 August and 24 October. All cobs were harvested on 16 October, weighed on 21 October, then dried, re-weighed, and counted on 13 November. Dry kernels were removed from cobs and weighed separately.

A final plant count was conducted on 24 October. Remaining aboveground crop biomass was collected from a 1.5 m section of the center row in each plot on 25 October. Popcorn plants were bundled and dried in a greenhouse, then weighed.

Figure 7. Wireworm sample collection

Statistical analysis

Plant emergence counts, height, and fresh and dry plant and fresh cob weight, and dry kernel weight data were tested for normality (Shapiro-WIlk) to ensure that ANOVA assumptions were satisfied and data were transformed as needed. ANOVA was used to test for treatment effects. Marginal means with standard errors were calculated for each treatment. All analyses were conducted using the jamovi interface for R, with a critical threshold of $\alpha = 0.05$ maintained throughout (The jamovi project 2021, R Core Team 2021).

Figure 8. Six week-old popcorn at experimental site.

Figure 9. 10 week-old popcorn at experimental site.

Results

Corn emergence was first observed on 10 July six days after seeding.

No treatment effect was detected on seedling survival (Fig. 10), plant height (Fig. 11), and dry kernel yield (fig. 12) (see appendix 2, 3).

Wireworms collected from the plots did succumb to *M. brunneum* infections in-situ: Wireworms (4 in total) were found in core samples from: 1C, 3B, 5A, 8A. 3 of the 4 wireworms developed mycelium (1C, 5A, 3B. Please see appendix section 2 for more details)(fig. 13, a moribund wireworm from replicate 3, treatment plot B) The infection in the wireworm in figure 13 was confirmed to be *M. brunneum* (Aaron Thien, pers. Comm.)

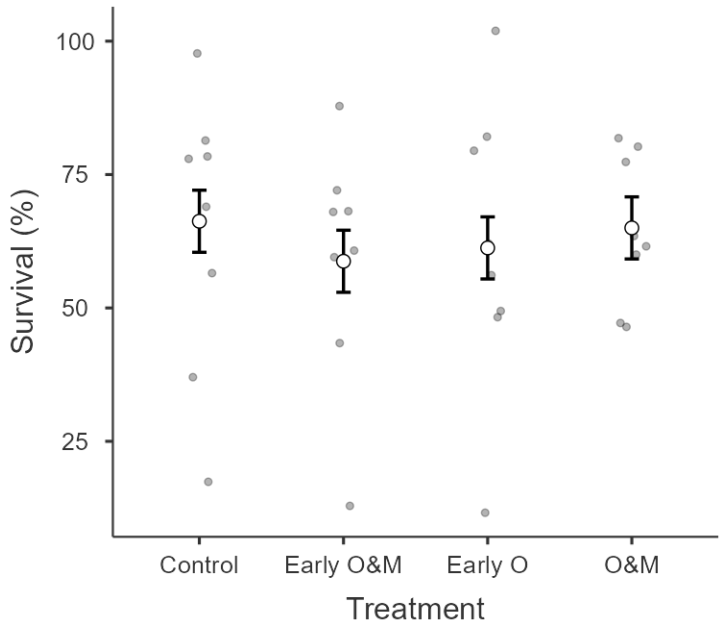

Figure 10. Popcorn survival rate by treatment on October 24th, 112 days after planting. Treatments were untreated controls (Control), rolled oats and *M. brunneum* applied 24 days before seeding (Early O&M), rolled oats applied 24 days before seeding (Early O), or rolled oats and *M. brunneum* applied at seeding (O&M). Error bars denote standard error of means (*n*= 8). Grey circles denote values for individual plots.

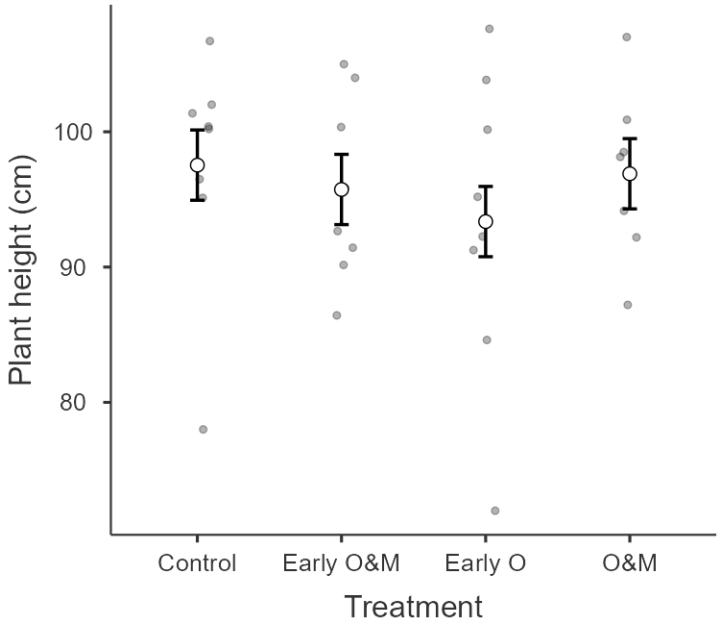

Figure 11. Popcorn plant height by treatment on October 24th, 112 days after planting. Treatments were untreated controls (Control), rolled oats and *M. brunneum* applied 24 days before seeding (Early O&M), rolled oats applied 24 days before seeding (Early O), or rolled oats and *M. brunneum* applied at seeding (O&M). Error bars denote standard error of means (*n*= 8). Grey circles denote values for individual plots.

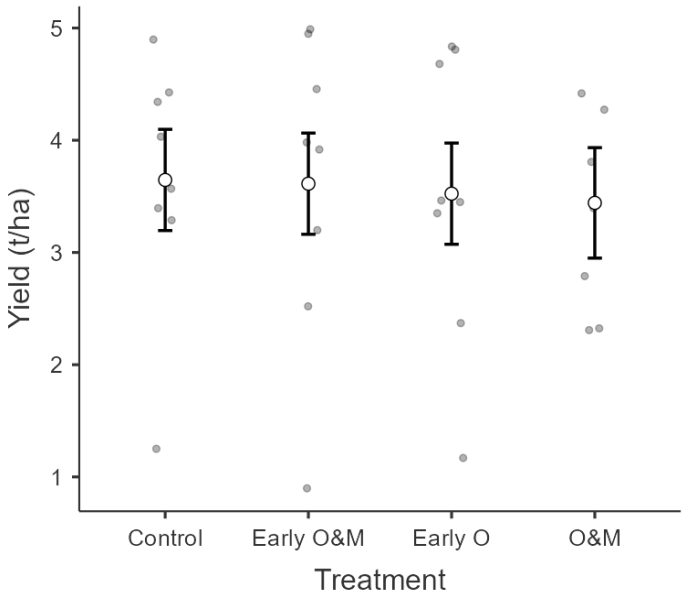

Figure 12. Dry popcorn yield by treatment. Treatments were untreated controls (Control), rolled oats and *M. brunneum* applied 24 days before seeding (Early O&M), rolled oats applied 24 days before seeding (Early O), or rolled oats and *M. brunneum* applied at seeding (O&M). Error bars denote standard error of means (*n*= 8). Grey circles denote values for individual plots.

Figure 13. Wireworm infected with *M. brunneum*, isolated from experimental plot

Discussion

The lack of significant treatment effects does not necessarily imply that *M. brunneum* was inactive. *M. brunneum* was applied to the same site in 2019, and may have had persistent effects in 2020. Pilz et al. (2011) report persistence of *M. anisopliae* in maize fields 15 months after application. Direct-seeded corn crops planted at this site failed due to wireworm pressure in 2015, 2017 and 2018 (Bomford, pers. comm.). Corn emergence and survival in all treatment plots in 2020 suggests reduced wireworm pressure, possibly due to residual effects of previous treatments; but this study was not designed to test for such effects.

Kabaluk et al. (2005) report that wireworms can detect, and avoid, soils with high concentrations of *M. brunneum* conidia. They are less likely to avoid conidia when food is present in the area. *M. brunneum* applied before seeding may have acted as a wireworm repellent.

The farm where the experiment took place has 11% organic matter, and plow-down of a rye cover crop before the beginning of the experiment likely added to the pool of labile C, generating CO_2 through microbial decomposition. These additional sources of CO_2 may have masked attractive effects of the decomposing rolled oats.

A number of questions arise:

- Does *M. brunneum* applied in prior growing seasons have any residual effect? If so, how long does an effect persist, and what conditions promote persistence?
- Does high soil organic matter content or high conidial density reduce the attractiveness of CO_2-producing lures like rolled oats?

These questions relate to the functional ecological relationships between *M. brunneum* and other agro-ecosystem components. Simultaneous management of multiple ecological factors may be necessary to ensure efficacy of *M. brunneum* as a biocontrol. These might be best understood through multivariate community analysis instead of single-factor experiments.

Future experiments could be Improved by digging treatment trenches along the entire length of the experimental area using a rotary plough and by applying conidia and rolled oats to treatment plots with an Earthway seeder.

Conclusion

No significant treatment effects were detected, despite the occurrence of *M. brunneum* in wireworms collected from the experimental site. Future research is needed to further elucidate the ecological interactions of *M. brunneum* within the agro-ecosystem, and to test for any residual effects due to repeated applications.

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Appendix 1 – Calculations

- Study area = 22.6 m x 10 m = 226 m^2
- Block area = 4.8 m x 5.3 m = 25 m^2
- Plot area = 2.25 m x 2.5 m = 5.6 m^2
- Row spacing = 0.75 m
- Row length = 2.5 m
- Plant spacing in-row = 0.15 m
- Plants
    - Per row = 2.5 m row length / 0.15 m plant spacing = 17 plants per row
    - For data collection: 17 plants in centre row – 2 guard plants at each end = 13 plants per plot
    - Guard: Two guard rows per plot and two guard plants on either end of each treatment row = 2 rows x 17 plants per row + 4 row end plants = 38 guard plants per plot
    - Per plot = 3 rows x 17 plants per row = 51 plants per plot
    - Total = 32 plots x 51 plants / plot = 1632 plants
- Four treatment bands per plot, spaced 0.75 m apart, between corn rows
    - Rolled oats
        - Per row = 10 g/m x 2.5 m/row = 25 g oats per row
        - Per plot = 4 rows per plot x 25 g/row = 100 g per plot
        - Total = 24 plots receiving oats x 100 g/plot = 2400 g of oats
    - *M. brunneum*, assuming 1.8 x 10^9 viable spores/g
        - Target rate = 10^{14} conidia/ha = 10^{10} conidia/m^2
        - Total treated area = study area / 2 = 226 m^2 / 2 = 113 m^2
        - Total *M. brunneum* application = 113 m^2 x 10^{10} conidia/m^2 = 1.13 x 10^{12} conidia

- Per plot = 1.13 x 10^{12} conidia / 16 treated plots = 7.1 x 10^{10} conidia per plot
- Per treatment row = 7.1 x 10^{10} conidia/m^2 / 4 rows = 1.7 x 10^{10} conidia per row
- Weight per row = 1.7 x 10^{10} conidia/row / 1.8 x 10^{9} viable conidia/g = 9.8 g/row
- Weight per plot = 9.8 g/row x 4 rows per plot = 39 g/plot
- Total weight = 39 g/plot x 16 treatment plots = 624 g

Appendix 2 - Field notes

June 10: all 32 Meta-oat and oat treatments applied.

July 16th: Soil cores taken from the northwest corners of all plots, within treatment bands. Soils samples were placed in brown paper bags and marked with the plot and rep they were found in.

Soil cores sifted using makeshift sifter made harvest bin and plastic mesh found on farm.

All wireworms placed immediately into Petri dishes with 5x5 cm pieces of moist disposable microfibre towels, packed into newspaper, and transported home by bicycle. All samples were given a slice of carrot upon arrival.

Wireworm observations:

Wireworms were found in core samples from: 1C, 3B, 5A, 8A

Observations of wireworms isolated from soil cores:

July 17th: No change

July 18th: 3B appears dead

July 19th: 5A has produced some kind of mycelium type growth. White, from between segments.<<<<<

July 20th: 3B has produced mycelium. White, from between segments. Some of 5A's mycelium has turned an olive green colour. 8A was also developing white mycelium from between segments.

July 22nd: 3B, half of mycelium on wireworm has turned from white to olive green. 5A has turned dark olive green. 8A has als turned dark olive green.

July 23rd: 4D appears dead, desiccated. 4A appears dead. 3B/5A/8A are all dark olive green now.

July 24: 4A moved slightly.

July 25: 2A, appears to have turned into a pupa, obviously not a wireworm.

July 28th: 1C activity reduced.

July 29th: 1C appears dead

July 31st: 1C white mycelium has appeared from between segments while lodged inside carrot slice.

Aug 1st: nominal

Aug 2nd: nominal

Aug 3rd: 1C more white mycelium

Aug 4-8: no change

Appendix 3
Raw Data

Rep	Treat	Survival (%)	Survival count	Yield (kg)	Height (cm)	Ears	Plant weight (kg)	Yield (kg/m)	Yield (t/ha)	Ears/m	Plant weight (kg/m)
1	Early O&M	40	4	0.56	93.6	24	0.72	0.37352	4.980267	16.008	0.72
1	Early O	50	5	0.54	84.6	18	0.92	0.36018	4.8024	12.006	0.92
1	O&M	50	5	0.26	87.2	12	0.83	0.17342	2.312267	8.004	0.83
1	Control	20	2	0.14	78.0	5	0.37	0.09338	1.245067	3.335	0.37
2	Early O&M	70	7	0.44	91.4	18	1.02	0.29348	3.913067	12.006	1.02
2	Early O	80	8	0.39	91.3	18	1.11	0.26013	3.4684	12.006	1.11
2	O&M	80	8	0.5	100.9	21	1.5	0.3335	4.446667	14.007	1.5
2	Control	60	6	0.55	102.0	21	1.3	0.36685	4.891333	14.007	1.3
3	Early O&M	60	6	0.5	90.2	22	0.99	0.3335	4.446667	14.674	0.99
3	Early O	10	1	0.13	72.0	7	0.07	0.08671	1.156133	4.669	0.07
3	O&M	50	5	0.26	92.2	10	0.49	0.17342	2.312267	6.67	0.49
3	Control	40	4	0.37	96.5	16	1.25	0.24679	3.290533	10.672	1.25
4	Early O&M	70	7	0.28	95.9	11	1.1	0.18676	2.490133	7.337	1.1
4	Early O	60	6	0.39	100.2	16	0.69	0.26013	3.4684	10.672	0.69
4	O&M	60	6	0.48	98.1	21	1.29	0.32016	4.2688	14.007	1.29
4	Control	80	8	0.45	100.4	18	1.54	0.30015	4.002	12.006	1.54
5	Early O&M	60	6	0.45	92.7		0.73	0.30015	4.002		0.73
5	Early O	50	5	0.27	95.2	14	0.89	0.18009	2.4012	9.338	0.89
5	O&M	60	6	0.38	98.5	17	1.32	0.25346	3.379467	11.339	1.32
5	Control	80	8	0.4	101.4	16	0.94	0.2668	3.557333	10.672	0.94
6	Early O&M	90	9	0.56	100.3	25	1.19	0.37352	4.980267	16.675	1.19
6	Early O	100	10	0.54	107.6	21	1.59	0.36018	4.8024	14.007	1.59
6	O&M	80	8		107.0	22	0.78			14.674	0.78
6	Control	80	8	0.38	95.1	18	0.97	0.25346	3.379467	12.006	0.97
7	Early O&M	10	1	0.1	104.0	3	0.2	0.0667	0.889333	2.001	0.2
7	Early O	60	6	0.53	103.8	20	0.79	0.35351	4.713467	13.34	0.79
7	O&M	60	6	0.43	94.1	16	0.84	0.28681	3.824133	10.672	0.84
7	Control	70	7	0.49	106.7	19	1.16	0.32683	4.357733	12.673	1.16
8	Early O&M	70	7	0.36	86.4	17	0.63	0.24012	3.2016	11.339	0.63
8	Early O	80	8	0.38	92.3	13	0.39	0.25346	3.379467	8.671	0.39
8	O&M	80	8	0.31	97.1	12	0.99	0.20677	2.756933	8.004	0.99
8	Control	100	10	0.5	100.2	16	1.18	0.3335	4.446667	10.672	1.18

Appendix 4 - Statistical Analysis

Randomized Complete Block Design

Design Properties

Treatments	Replicates	Plots	ID
4	8	32	5827

Degrees of Freedom

Source	DF
Blocks	7
Treatments	3
Residual	21
Total	31

ANOVA

ANOVA - Plant fresh weights Kg (no cobs):

	Sum of Squares	df	Mean Square	F	p
Reps.	5.22	7	0.746	2.09	0.091
Treatment	1.31	3	0.437	1.22	0.326
Residuals	7.50	21	0.357		

[3]

Assumption Checks

Normality Test (Shapiro-Wilk)

Statistic	p
0.970	0.499

Post Hoc Tests

Post Hoc Comparisons - Treatment

Comparison			Mean Difference	SE	df	t	P_{tukey}
Treatment		Treatment					
A	-	B	0.281	0.299	21.0	0.941	0.783
	-	C	−0.104	0.299	21.0	−0.347	0.985
	-	D	−0.276	0.299	21.0	−0.924	0.792
B	-	C	−0.385	0.299	21.0	−1.288	0.580
	-	D	−0.558	0.299	21.0	−1.865	0.273
C	-	D	−0.173	0.299	21.0	−0.577	0.938

Note. Comparisons are based on estimated marginal means

Estimated Marginal Means - Treatment

			95% Confidence Interval	
Treatment	Mean	SE	Lower	Upper
A	1.81	0.211	1.37	2.25
B	1.53	0.211	1.09	1.97
C	1.91	0.211	1.47	2.35
D	2.08	0.211	1.64	2.52

ANOVA

ANOVA - Cob fresh Yields (Kg)

	Sum of Squares	df	Mean Square	F	p
Reps.	0.2595	7	0.03707	0.688	0.681
Treatment	0.0258	3	0.00859	0.159	0.922
Residuals	1.1314	21	0.05387		

[3]

Assumption Checks

Normality Test (Shapiro-Wilk)

Statistic	p
0.967	0.422

Post Hoc Tests

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Post Hoc Comparisons - Treatment

Comparison							
Treatment		Treatment	Mean Difference	SE	df	t	p_{tukey}
A	-	B	0.06875	0.116	21.0	0.5924	0.933
	-	C	0.03625	0.116	21.0	0.3124	0.989
	-	D	0.00125	0.116	21.0	0.0108	1.000
B	-	C	−0.03250	0.116	21.0	−0.2800	0.992
	-	D	−0.06750	0.116	21.0	−0.5816	0.937
C	-	D	−0.03500	0.116	21.0	−0.3016	0.990

Note. Comparisons are based on estimated marginal means

Estimated Marginal Means - Treatment

			95% Confidence Interval	
Treatment	Mean	SE	Lower	Upper
A	0.684	0.0821	0.513	0.854
B	0.615	0.0821	0.444	0.786
C	0.647	0.0821	0.477	0.818
D	0.682	0.0821	0.512	0.853

ANOVA

ANOVA - 3rd height measurements (cm)

	Sum of Squares	df	Mean Square	F	p
Reps.	1048.9	7	149.8	3.730	0.009
Treatment	96.6	3	32.2	0.802	0.507
Residuals	843.6	21	40.2		

[3]

Assumption Checks

Normality Test (Shapiro-Wilk)

Statistic	p
0.928	0.035

Assumption Checks

Normality Test (Shapiro-Wilk)

Statistic	p
0.928	0.035

Post Hoc Tests

Post Hoc Comparisons - Treatment

Comparison							
Treatment		Treatment	Mean Difference	SE	df	t	p_{tukey}
A	-	B	0.951	3.17	21.0	0.300	0.990
	-	C	−2.585	3.17	21.0	−0.816	0.846
	-	D	−3.223	3.17	21.0	−1.017	0.741
B	-	C	−3.536	3.17	21.0	−1.116	0.684
	-	D	−4.174	3.17	21.0	−1.317	0.563
C	-	D	−0.638	3.17	21.0	−0.201	0.997

Note. Comparisons are based on estimated marginal means

Estimated Marginal Means - Treatment

			95% Confidence Interval	
Treatment	Mean	SE	Lower	Upper
A	94.3	2.24	89.7	99.0
B	93.4	2.24	88.7	98.0
C	96.9	2.24	92.2	101.6
D	97.5	2.24	92.9	102.2

ANOVA

ANOVA - Survival (%)

	Sum of Squares	df	Mean Square	F	p
Reps.	9272	7	1324.6	4.888	0.002
Treatment	284	3	94.8	0.350	0.790
Residuals	5691	21	271.0		

[3]

Assumption Checks

Normality Test (Shapiro-Wilk)

Statistic	p
0.949	0.137

Post Hoc Tests

Post Hoc Comparisons - Treatment

Comparison							
Treatment		Treatment	Mean Difference	SE	df	t	p_{tukey}
A	-	B	−2.50	8.23	21.0	−0.304	0.990
	-	C	−6.25	8.23	21.0	−0.759	0.872
	-	D	−7.50	8.23	21.0	−0.911	0.799
B	-	C	−3.75	8.23	21.0	−0.456	0.968
	-	D	−5.00	8.23	21.0	−0.607	0.929
C	-	D	−1.25	8.23	21.0	−0.152	0.999

Note. Comparisons are based on estimated marginal means

[4]