**Oomycete community response from oomicide seed treatments**

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**Abstract**

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**Introduction**

Soybean seeds and seedlings are vulnerable from a number of soil borne pests and pathogens and protection of developing soybeans is important. Cultural techniques like tillage can help reduce pressure from some soil borne pathogens like *Pythium* and *Phytophthora*. However, leaving crop residue in the field has become a popular agronomic practice to reduce soil erosion and build soil organic matter. Residue can harbor overwintering inoculum of oomycete pathogens which can attack germinating seeds and developing seedlings. Additionally, growers are planning earlier into cooler wetter soil which exposes seeds and developing seedlings to conditions favorable for infection from oomycete pathogens. Besides *Phytophthora sojae*, soybean varieties are not generally bred for resistance to or are known to have resistance to a diversity of oomycete pathogens like *Pythium* or *Phytopythium* (Bradley 2008; Rupe et al. 2011). Soybean associated oomycetes vary in pathogenicity and virulence (Rojas et al. 2016a; Broders et al. 2007), and oomycete communities can vary across location, temperature and edaphic gradients (Rojas et al. 2017b; Broders et al. 2009). This diversity and complexity within a community make it difficult to manage root and stem rot pathogens since management decisions must be made on a community level. Therefore, anti-oomycete fungicides (oomicides) that are efficacious on a number of oomycete species are important management tools. Oomicides are usually applied to the seed coat prior to planting to protect seeds and developing seedlings.

Phenylamide fungicides, including metalaxyl and mefenoxam, are commonly used

to target oomycetes. In 2011, phenylamide sales accounted for approximately 405 million U.S. dollars (Gisi and Sierotzki 2015). Metalaxyl, or its active stereoisomer metalaxyl-M (mefenoxam) inhibits ribosomal RNA synthesis, specifically RNA polymerase complex I (Davidse 1995). Metalaxyl was first registered for use in the U.S. in 1979, and practical resistance, or loss of fungicide efficacy in the field to metalaxyl or mefenoxam are major problems in the U.S. and is a concern when used as the sole oomicide in seed treatments (Gisi and Sierotzki 2015; Taylor et al. 2002). However, seed treatments can contain multiple oomicides. For example, ethaboxam is a thiazole carboxamide belonging to the benzamide fungicide class, registered for use on oomycete pathogens (Kim et al. 1999). In 2014, ethaboxam was registered in the U.S. for use as a seed treatment on corn and soybean. The mode of action is microtubule formation disruption (Uchida et al. 2005). Inter- and intraspecific variation to both mefenoxam and ethaboxam have been reported (), therefore efficacy of a seed treatment can depend on the types and rates of oomicides applied to the seed. For example, some *Pythium* lineages inherently contain a C239S mutation in β-tubulin which confers insensitivity to ethaboxam. Therefore, if ethaboxam is used as the sole oomicide on seed treatments, it may select for certain *Pythium* spp. which are insensitive to ethaboxam. Although field efficacy and *in vitro* studies have been performed the extent to which oomicide containing seed treatments directly reduce oomycete colonization or alter species distributions on soybean roots is understudied. Also, it not well documented if isolates obtained from soybean roots that contained oomicides are more insensitive to the oomicide the seed was treated with.

The objectives of this manuscript were to isolate oomycetes from soybean roots that were non-treated, treated with mefenoxam or mefenoxam and ethaboxam to (1) understand the degree to which seed treatments reduce oomycete infection, (2) determine the extent to which seed treatment alter oomycete diversity on soybean roots, and (3) determine inter- and intraspecific variation in sensitivity to mefenoxam or ethaboxam.

**Materials and Methods**

**Field sites description.** In 2016 non-treated soybeans (AG2233), soybeans treated with mefenoxam (Apron XL®), or soybeans treated with mefenoxam + ethaboxam (Intego Suite®) were planted in Ohio, Michigan, Illinois, Iowa and Indiana. In 2017 soybeans with the same treatments as described above were planted in the same locations except for Indiana. In Ohio, Illinois, Iowa, and Indiana plots were arranged in strips and whole plants were sampled in a stratified random method such that each treatment was broken into three equally spaced strata. Twenty-one plants from each strata were collected at V1-V2 and sent to Michigan State University for isolation. In Michigan, plots were arranged in a completely randomized block design with six replicates. X plants were sampled randomly from each plot. Soil was collected at each field site and submitted to the Michigan State University Soil and Plant Nutrient Laboratory for analysis of physical and chemical properties (Table 1).

**Oomycete isolation.** Oomycetes were isolated from root and stem tissue as described in Rojas et al. (2017a). Briefly, roots were rinsed with tap water for at least 30 minutes to remove soil. Then a portion of lateral root tissue, approximately 3.8 to 5 cm, and a 1.5 cm portion of taproot was placed onto corn meal agar medium (CMA) amended with PARPB (pentachloronitrobenzene [PCNB] 50 mg L-1, ampicillin 250 mg L-1, rifampicin 10 mg L-1, pimaricin 5 mg L-1, and benomyl 10 mg L-1) (Jeffers 1986). Pure cultures were obtained via hyphal tip isolation.

**Oomycete identification.** Oomycetes were identified by sequencing the internal transcribed spacer (ITS) region of the rDNA. In 2016, five plugs of cultures growing on CMA+PARPB, were grown in 10% V8 broth containing 0.5 g CaCO3. Mycelia were harvested and lyophilized overnight. Approximately 25 – 30 mg of lyophilized tissue was ground using a FastPrep FP120 (Thermo Fisher Scientific, Waltham, MA) and genomic DNA (gDNA) was extracted using the DNeasy plant Mini Kit (Qiagen Sciences Inc., Germantown, MA, USA) or OMEGA Mag-Bind Plant DNA Plus kit (Omega Bio-Tek, Norcross, GA, USA) by following the manufacturer’s instructions. In 2017, one plug of cultures growing on CMA+PARPB were grown in 1 ml of 10 % V8 broth within a 2 ml 96 well plate. Porous sealing tape was applied to the 96 well plate to allow gas exchange and avoid contamination. After seven days, the plate was frozen at -20ºC and lyophilized overnight. Following lyophilization, one sterile 6 mm ball bearing was added to each well along with 100 μl extraction solution. Extraction solution consisted of a final solution of 0.5 M Tris, 0.25 M KCl, 1 x 10-3 M EDTA and pH 9.5-10. Lyopholized mycelium was ground within the 96 well plate using a GenoGrinder (blah blah) at 1000 rpm for 30 seconds. Following grinding within the extraction solution the 96 well plate was incubated at room temperature for 10 minutes followed by incubation for 10 minutes at 95ºC. Then 300 μl of 3 % bovine serum albumin (BSA) solution was added to each well of the 96 well plate.

PCR reactions consisted of 1X DreamTaq buffer containing 1.5 mM MgCl2, 0.2 mM dNTP, 0.5 U of Dreem polymerase, 0.2 μM ITS6 and ITS4 primers and either 1 μl of 1:10 diluted genomic DNA or 2 μl of the solution in 96 well plates. Thermal cycling conditions were as followed: 98ºC for 5 min followed by 35 cycles of 98ºC for 1 min, 60ºC for 45 seconds and 72ºC for 1.5 min followed by a final extension at 72ºC for 7 min. Amplicons were checked on a 1.5% agarose gel and amplicons subsequently purified by adding 5 μl containing 3 U exonuclease I and 0.5 U shrimp alkaline phosphatase (Thermo Scientific) and incubating at 37ºC for 45 min followed by an enzyme inactivation at 85ºC for 10 min. Amplicons were sanger sequenced via Macrogen USA (Rockville, Maryland) or at the Michigan State University Genomics core. Forward and reverse sequences were trimmed and assembled with Codon Code Aligner v4.2.7 and consensus sequences were compared against curated set of oomycete sequences (Robideau et al. 2011) using BLAST. Sequences with BitScore > 1000 and a sequence identity > 97 % were assigned to a species (Rojas et al. 2017a).

**Fungicide sensitivity.** The sensitivity to mefenoxam or ethaboxam for X isolates was carried out using the method of Noel et al. (2018 *in review*). Technical grade ethaboxam was dissolved in 99.5 % acetone. Mefenoxam was dissolved in dH20. Isolates were tested in amended dilute V8 broth medium containing ethaboxam or mefenoxam concentrations 0, 0.01, 0.1, 1, 10 and 100 μg ml-1. Medium amended with 0.0995 % (v/v) acetone was used for the control. The optical density at 600 nm (OD600) for each isolate was transformed to percent relative growth by dividing the mean optical densityof each fungicide concentration by the mean OD600 without fungicide, multiplied by 100. The EC50 (effective concentration to reduce OD600 by 50 % when compared to the non-amended control) was estimated for each isolate.

**Statistical Analysis.** All statistical analysis was carried out in Rv3.3.2 (R Core Team 2018). To test the effect of seed treatment on isolation success or failure a generalized linear model using a blah blah distribution was used to model count data for each seed treatment. To test if the total number of isolates per treatment was different the average number of isolates per treatment was subjected to a three-way analysis of variance (ANOVA) treating seed treatment, tissue, location and year as fixed effects. To test if seed treatment reduced oomycete richness or evenness, a one-way ANOVA was conducted on the Shannon diversity index within the “vegan” package of R. To test if seed treatment altered community structure permutational multivariate analysis of variance (PERMANOVA) was carried out following non-metric multidimensional scaling ordination on Bray-Curtis distances using the “adonis” function in “vegan”. Dose response analysis was carried out with the “drc” package in Rv3.3.2 (Ritz and Streibig, 2015). Percent relative growth was modeled against log transformed fungicide doses using a four-parameter log-logistic model. The absolute EC50 was estimated by solving for the concentration where 50 % inhibition occurred (Noel et al. 2018b).

1. Field sites and description
   1. Location
   2. Soil properties
2. Isolation of oomycetes
   1. CMA+PARPB
   2. Stem and root tissue
3. Identification of oomycetes
   1. 2016 – DNA extraction with Omega plant DNA kit
   2. 2017 – Quick extraction protocol
   3. PCR protocol with ITS
   4. Sanger sequenced
   5. Identification process
4. Fungicide sensitivity testing
   1. Brief methods on HTFS
   2. Analysis
5. Statistical analysis
   1. Count data for isolation success and failures
   2. Community data
6. Intro on soybean production and influence of oomycetes
   1. ~~Soybeans account for X $ and %~~
   2. ~~Protecting them from pathogens is important~~
   3. ~~Resistant varieties are not always available~~
   4. ~~Fungicides are important management tools~~
   5. ~~Pythium is a major threat~~
   6. ~~Oomicides are important~~
7. Fungicide resistance development theory and how it applies to Pythium communities
   1. Fungicides select for tolerant isolates in populations either discretely or multistep methods
   2. Pathogen factors for resistance development
   3. Fungicide factors for resistance development
   4. Seed treatments are low risk
   5. Pythium low risk
   6. Important where natural inherent resistance is prevalent
      1. Ethaboxam vs mefenoxam story
   7. Often why chemistries are mixtures on seed treatments?
8. Conditions that influence Pythium communities (Rojas et al; Zitnick et al; Broders et al)
   1. Moisture
   2. Edaphic factors
      1. Clay content
      2. Soil carbon availability
   3. Location
   4. Not explored enough – Fungicides
      1. Direct vs indirect effects
      2. Non-target effects
      3. Are those with natural resistance increased?
      4. Does adding fungicide mixtures significantly alter the community at a phenotype and composition level?
         1. For example, repeated use of a chemical will select for tolerant genotypes at the population and community level.
   5. Objectives and general methods
      1. The objectives of this manuscript were to
         1. Isolate oomycetes from soybean roots treated with different fungicide mixtures
         2. Does the presence of oomicides decrease isolation success
         3. Do different fungicide mixtures alter the community at the population or community level?
      2. To do this we
         1. Field sites were set up where the same seed treatment was planted in the same location for two years
         2. Isolated oomycetes from soybean roots treated with different oomicide seed treatments in 2016 and 2017
         3. Tested fungicide sensitivity to ethaboxam and mefenoxam
         4. Tested if community sensitivity to these chemicals decreased based on the chemistries used.

**Materials & methods**

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5. Statistical analysis
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   2. Community data

**Results**

1. Distribution of species at each location (figure 1)
2. Root and stem isolation data species distribution
3. Count data tables
4. Fungicide sensitivity data (EC50 data, distributions and comparisons)
5. Growth rate data vs abundance?
6. Phylogeny stuff? Make phylogeny based on the consensus between ITS sequences of species and present abundance data that way?
7. Increase/decrease in species abundance (Alpha diversity with seed treatment and location)
8. Indicator species analysis with seed treatment by location?
9. Beta diversity (NMDS plots, bray Curtis distance, Jaccard distance)

**Discussion**

1. Overview paragraph
   1. Sentence of summary of the objective
   2. Sentence summary of methods
   3. Significant findings
      1. Planting soybeans with the same seed treatments two years in a row did not decrease sensitivity to mefenoxam or ethaboxam
      2. The presence of a seed treatment did reduce the number of isolates obtained from those roots
      3. Community composition was strongly influenced by location but seed treatment had subtle effects
2. Paragraph on resistance development theory
   1. Seed treatments low risk, differences in species sensitivity rather than within population seem more important for oomycete communities in soybean associated oomycetes
   2. Because of this there is potential for selection/filtering of certain species due to repeated use of ethaboxam. However, not able to directly test this in this study.
   3. The addition of ethaboxam reduced some taxa but was not significantly different than with mefenoxam alone.
   4. Less risk of resistance development with ethaboxam mixture
3. Paragraph on seed treatment efficacy
   1. Yup seemed like they worked
4. Paragraph on community composition
   1. Location a strong driving factor – differences in soil characteristics and such
   2. Seed treatment seemed to provide little community separation
      1. Plant age and questions of seed treatment effectiveness over time.
   3. Community assembly on treated seed
      1. Competition between species
      2. Fast vs. slow growing species and their presence on seed treatments
5. Concluding paragraph
   1. Future research
      1. Utilizing and improving oomycete sequencing for increased throughput and sampling
      2. Phenotypes can be important!
   2. Conclusion