**Final Project QMEE**

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**Introduction**

The crop establishment is key factor for soybean growers at the beginning of the season where most of the time, seedlings are subject of the attack of soilborne pathogens, resulting in damping off and poor plant establishment. Soybean seedling diseases impact the plant root system at the beginning of the season, however the root infections can occur at later stages, impacting crop physiology and yields. In 2005, seedling diseases caused losses around 829 tons in the US (Wrather & Koenning, 2006). Different factors could influence the outcome of the interaction of soilborne pathogens and soybean plants, such as seed genetic background, the climate, edaphic factors and their interaction with the soil inhabitants (Broders et al., 2009). These soilborne diseases have been attributed to several pathogens most of them fungi and oomycetes (fungi-like organisms). However, the key species playing a role in disease are not fully known (Broders et al., 2007).

In fact, the oomycetes *Pythium* and *Phytophthora* are two of the main causal agents of soybean root diseases, for instance losses have increased by four-fold in the last ten years (Koenning & Wrather, 2010). The increased incidence is related to some of cultural practices now being used by growers, like no-till and early planting. These practices and the environment interact strongly with the microbial communities present in the soil, causing shifts in the different species that exist in this ecosystem (Arcate et al., 2006). The knowledge of the diversity of species causing soybean seedling diseases in the US Midwest is limited. We initially utilized a culture-based approach to start understanding the diversity of oomycetes associated with soybean seedling diseases in the US sampling in 2011 and 2012. A semi-selective medium was selected to increase the recovery rate of a group of organisms, in this case Oomycetes. The identity of the species was confirmed based on the sequencing of Internal Transcribed Spacer (ITS) of the ribosomal DNA.

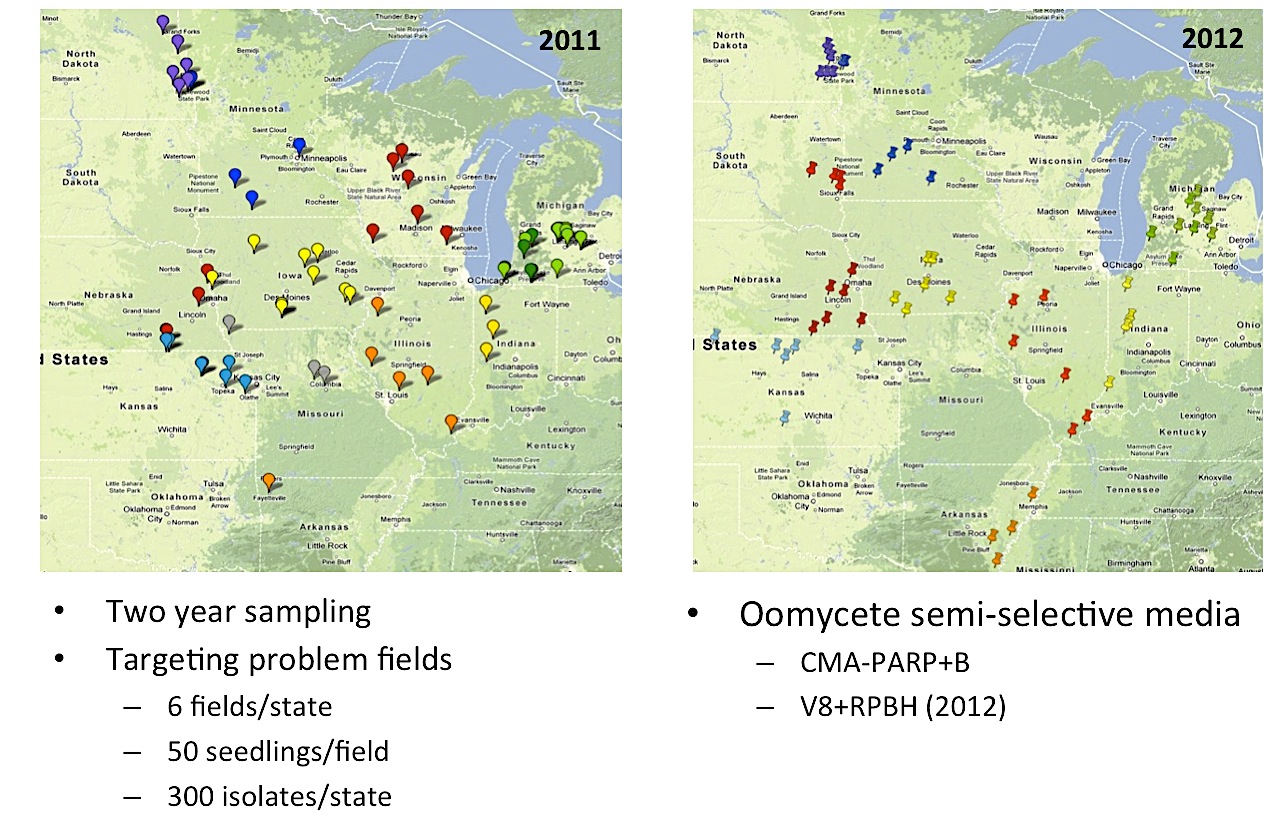
Oomycetes are known to be aggressive plant pathogens, but the number of species associated with different plant species or different ecosystems is not well known. Recent studies have described multiple new species based on soil and water systems surveys (Kang et al., 2010). Different studies have been done co-relating environmental data with fungal species distribution, resulting on the identification of different drivers such a soil properties and latitude (Tedersoo et al., 2014). However, the relationship of species distribution and environmental factors is undetermined for the oomycetes. The research question focuses on what are the oomycete species associated with soybean seedling diseases. Our hypothesis is that seedling diseases are cause by multiple oomycete species, which some of this species could be acting as pathogen complexes to cause disease across the Midwest. In addition, we also hypothesize that there are climatic and edaphic factors driving the presence and abundance of these oomycetes on the different fields.

The study of the diversity of oomycete pathogens associated to crops like soybean will provide a better understanding of the complexes of organisms that might be associated with disease under specific conditions, for instance climatic and edaphic variables. In order to conduct the analysis different linear models will be evaluated to disentangle the role of different factors on oomycete diversity, also including geographical variables such as latitude to evaluate the hypothesis of diversity on relation to distance from the equator. The understanding of microbial communities associated with soybean will be significant since it is major crop with economical importance not only as staple food, but also as biofuel sources to reduce the impact of potential pathogens, as we understand their distribution, frequency and abundance.

**Methods**

***Sample collection, species ID and climatic data***

Root rot symptomatic soybean seedlings were collected from fields with a history of plant establishment issues and damping-off. A total 50 symptomatic seedlings were collected in W-shaped transect in a field (replication unit). Within a state, 6 fields were sample and a total of 10 to 12 states were sampled in two years 2011 and 2012 (Figure 1). Thus, a hierarchical sampling was conducted collecting plants within single fields within states in the Midwest.



**Figure 1.** Soybean fields sampled across the Midwest during 2011 and 2012.

Symptomatic soybean seedlings were washed with tap water, air-dried and isolations were done placing root tissue on CMA+PARP semi selective media. Plates were incubated at room temperature and visually inspected for hyphal growth after 3 days post isolation. Hyphal tips were transferred to clean media and pure cultures were stored and grown for DNA extraction. DNA extractions were conducted at the genomics center (RTSF – MSU) using a phenol-chloroform automated system (AutoGen Inc., Holliston, MA). Isolates were identified by amplification and sequencing of the Internal Transcribed Spacer (ITS) of the rDNA using primers ITS6 and ITS4 (Cooke et al., 2000). Sequences were assembled using CodonCode Aligner (CodonCode Corp., Dedham, MA, USA) and corroborated against a local database.

Climatic data was collected based GIS coordinates for each sample location and used to query different climatic databases to obtain different environmental parameters. Data was obtained from the database PRISM (<http://www.prism.oregonstate.edu/>) as shape files, and it was imported into DIVA-GIS (<http://www.diva-gis.org/>) for their correlation and extracting climatic parameters for each of the specific locations included in this study.

***Statistical methods***

*Data exploration*

A dataset was constructed based on number of species identified and diversity indices. In addition, based on latitude and longitude also included in the dataset, climate data such as temperature and precipitation were collected. Data was initially explored to determine the transformation of the data or exclusion of data. The field corresponding to Arkansas collected on 2011 was removed from the data due to issues during data collection since only one field was sampled, and it was oversampled deviating from methods followed in other fields. Parameters that contain quantitative data, such as temperature, precipitation and latitude were centered to reduce possible issues with colinearity (Table 1). These parameters were centered and added to the data to explore data and construct models. As part of the exploration, plots of different parameters were examined against operational taxonomic units (OTU) or Shannon index.

**Table 1.** List of parameters utilized to build the different models in this study.

|  |  |  |  |
| --- | --- | --- | --- |
| **Parameter** | **Class** | **Data Type** | **Biological Importance** |
| Operational Taxonomic Unit (OTU) | Numeric | Absolute | Sequence based definition of organisms, for instance based on 97% similarity |
| Shannon diversity index | Numeric | Absolute | Diversity index based on the formula H’= |
| Latitude | Numeric | Interval | Distance fro the equator, it has been shown to correlate with species diversity |
| Temperature | Numeric | Interval | This parameter could affect growth rate and niche of the different oomycete species |
| Precipitation | Numeric | Interval | Accumulated water could play an important role in disease, since it could enhance spore germination |
| Year | Factor | Nominal | Temporal variation of species, also dependent on weather conditions |

*Linear model and model selection*

Different linear models were constructed with two different response variables in order to determine the best fitting model to the oomycete diversity data. The goal is to determine which one of the responses is better to explain the diversity of these organisms, thus we can predict their distribution and associate this data with pathogenicity of different species. Initially, climatic variables were included in the different models in order to determine how well these parameters explain the diversity of the oomycete species. In order to preselect among different models, we initially propose multiple models for both responses: Shannon diversity index (Table 2) and OTU (Table 3). All of the models developed for these two response variables were evaluated using Akaike Information Criterion (AIC), the Log Likelihood, delta AIC and adjusted R2.

**Table 2.** Models preselected for Shannon index as response variable.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Model** | **Equation** | **Parameters** | **AIC** | **Delta AIC** | **Log Likelihood** | **Adj.**  **R2** |
| Shannon ~ Latitude |  | 2 | 227.9 | 0.0 | -110.99 | 0.056 |
| Shannon ~ Latitude + Temperature |  | 3 | 229.9 | 1.8 | -110.99 | 0.048 |
| Shannon ~ Latitude + Temperature + Latitude\*Temperature |  | 4 | 231.9 | 2.0 | -110.95 | 0.039 |
| Shannon ~ Latitude + Precipitation + Latitude\*Precipitation |  | 4 | 231.6 | 2.0 | -110.79 | 0.042 |

**Table 3.** Models preselected for OTUs as response variable.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Model** | **Equation** | **Parameters** | **AIC** | **Delta AIC** | **Log Likelihood** | **Adj.**  **R2** |
| OTU ~ Temperature |  | 2 | 805.6 | 0.0 | -399.80 | 0.024 |
| OTU ~ Latitude + Precipitation + Latitude\*Precipitation |  | 4 | 805.5 | 2.0 | -397.73 | 0.042 |
| Shannon ~ Latitude + Precipitation |  | 3 | 805.6 | 2.0 | -398.82 | 0.032 |

The candidate models were evaluated based on different statistical parameters under the general linear model, in order to establish their fitting. Coefficient plots and autocorrelation plots were examined to determine if data was problematic, and to determine the colinearity or other issues that could be affecting the use of the linear models. Two best fitting models were selected based on the lack of correlation and also based on the complexity of the models for further analysis. These models were further evaluated using variance inflation factors (VIF) were calculated for both models, and analyzed using the rule of thumb of VIF>10 to determine the existence of issues in the model. In addition, diagnostic plots were generated for both models under the two different responses.

*Bootstrap residual resampling for parameters*

In order to establish confidence intervals for parameters included in the models selected before, a bootstrap analysis was conducted on the residuals, having in mind the determination of biases of the data collected during the sampling. In this case, we assuming that temperature, latitude and precipitation are fixed, and there limited error in their measurements. The bootstrap analysis was conducted on the two models using Shannon diversity index and OTU number respectively. In this case a total 5000 replications were conducted to determine confidence intervals using bootstrap function.

*Power analysis*

Based on the models previously analyzed and considering the coefficients for these models, we used power analysis to determine the sample size required to have enough power to make any biological inferences based on the model that have been selected. For this approach a monte carlo simulation was conducted using the coefficient of the models, in this case the intercept and assuming a slope that ranges from 0 to 1. A total of 1000 replicates were conducted, increasing sample size by 20 in a range of 10 to 200 samples. Results were plotted as contour plots.

*Mixed models*

The best fitting model were reevaluated using mix model to account for the differences on sampling between years, since conditions, specifically climate was quite divergent. A covariance structure based on the factor year was considered under the simple covariance method known as compound symmetry. The models were re-evaluated using AIC.

**Results**

The diversity of the oomycetes across the soybean producing area ranges from mostly from 5 to 20 species per field if we consider the OTU parameter. The diversity index actually suggests a really tight diversity across the entire set of fields sampled. Nonetheless, we have to consider that we are using a very traditional approach to capture some of the diversity present in the field, and we are limited by number of strains that we can recover from infected plants or the soil. In this case, we are using culture-based technique and the set of species that could be recover is probably limited due to different biases. Thus, when this information on the number of species present per field or the diversity tries to be correlated with other parameters, the models are going to be limited to the data that we have available.

In this case, Shannon diversity index is capturing the alpha diversity which a diversity pertaining only to the sample itself, which could be missing other important factors like the differences between communities at two different locations, in this case we are talking about beta diversity. However, we hypothesize that there was a correlation between diversity per field in relation to other factors like climate. In this case metadata was collected using GIS coordinates to construct models based on some climate parameters. Using this information and climate data, linear models were constructed to determine if there was a correlation of the species observed at one field with these parameters. This question will help us to understand what environmental factors could drive the presence and abundance of some species at certain locations.

However, in general the models had limited correlation to explain the species observed based on temperature, precipitation and latitude.

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