**Nebraska Soybean Board**
**FINAL Research Report Form**

DS VB

1/13/2020

Note: Submit this report no later than 90 days after the NSB-funded project officially terminates.
This post-project 90-day time-frame will allow the Lead PI time to complete any final data analysis and a final technical report, plus the drafting of any articles for submission to scientific journals. Note that this completed report will be provided to the National Soybean Checkoff Research Database, (soybeanresearchdata.com).

***Project # and Title:*** #1720 Fungicide Resistance in Rhizoctonia solani and Implications for Soybean Fields in Nebraska

***Principal Investigator:*** Sydney Everhart, Department of Plant Pathology, UNL

***Co-PI's & Institutions:*** Anthony Adesmoye, formerly at Department of Plant Pathology, UNL, and current affiliation is Terramera, Vancouver, CA

***Project Date (Including Extension):*** 10/01/2015 ***to*** 09/30/2019 ***(For example: mm/dd/yyyy to mm/dd/yyyy)***

***Total Budget for Project:*** $ 121,961.00

***1. Briefly State the Rational for the Research:***

Nationally, Rhizoctonia solani is one of three pathogens most commonly associated with soybean seedling disease, where fungicide seed treatments are the recommended line of defense. However, prior to our research, there was no information about the species of Rhizoctonia causing soybean seedling diseases in Nebraska, nor was there information about fungicide efficacy. Thus, our research sought to fill that gap and yielded novel insights into the species of Rhizoctonia that are most common in Nebraska and shown that some fungicides are ineffective. This was a collaborative research project between the Everhart and Adesemoye labs. Two doctoral students (S. Kodati and N. Gambhir) are being trained in soybean disease management using laboratory and molecular techniques and these projects are part of their dissertation research (S. Kodati graduated fall 2019 and N. Gambhir graduates spring 2020).

***2. Research Objectives: (copy from project, but keep in a brief bullet format)***

Our objectives were to:
1. Perform a comprehensive survey of soybean fields for R. solani;
2. Determine fungicide sensitivity of each isolate to selected commercial fungicides;
3. Identify the most prevalent anastomosis groups (within-species subgroups);
4. Develop and apply genetic markers to characterize the structure of populations within the two most prevalent R. solani subgroups.

***3. General Approach Used and (if applicable) the Nebraska Test Locations:***

A survey of soybean fields was conducted to determine the Rhizoctonia species / sub-species responsible for causing Rhizoctonia seedling disease in Nebraska. An average of 18 soybean fields per year were in 2015, 2016, and 2017 and a grand total of 957 soil and plant samples were collected. From these, more than 115 Rhizoctonia were isolated and identified to species / sub-species using gene sequencing. Our results showed that R. zeae and Rhizoctonia solani AG-4 were the two most common in Nebraska. We identified R. zeae to be the most widespread and prevalent species in Nebraska, so further analysis was done on this species. The efficacy of four different seed treatment fungicides in controlling R. zeae was tested using laboratory and greenhouse techniques. We used whole-genome sequencing of a selection of R. zeae isolates and then bioinformatic approaches to identify candidate Simple Sequence Repeat (SSR) loci for marker development. Genotyping population structure of R. zeae in Nebraska.

***4. Describe Deliverables & Significance Attained for Each Research Objective:***

(Obj. 1) – An average of 18 soybean fields per year were in 2015, 2016, and 2017 and a grand total of 957 soil and plant samples were collected. From these, more than 115 Rhizoctonia were isolated and identified to species / sub-species using gene sequencing. We also optimized laboratory methods for isolation and growth of Rhizoctonia, which will enable future studies to use standardized approaches to study this pathogen. Isolates from this objective were the focus of studies in Objs. 2, 3, and 4.

(Obj. 2) – Our laboratory studies showed current commercial fungicides (prothioconazole, sedaxane, and fludioxonil) are effective and no fungicide resistance was observed. However, a crucial finding was that R. zeae is completely insensitive to azoxystrobin fungicide (both in the lab and in the greenhouse), which is currently one of most common fungicides used owing to its expected high specificity of action. Thus, any use of azoxystrobin is not expected to have an effect on R. zeae.

(Obj. 3) - Rhizoctonia zeae and R. solani AG-4 were determined to be the two most prevalent groups in Nebraska, which is different than what a recent study in Illinois found. Our greenhouse studies showed that R. zeae is able to cause disease on soybean seedlings. Although we observed no change in stand count, infected seedlings had reduced plant biomass. Young plants infected with R. zeae tend to be weaker, which would increase susceptibility to other pathogens in field conditions. Since the two Rhizoctonia spp. most widely prevalent in Nebraska are different from what has been documented in other states (MN, IL, OH, and AR), disease management recommendations from other states may need to be revised.

***4. Describe Deliverables & Significance Attained for Each Research Objective (continued)***

(Obj. 4) – Forty-three primers have been designed for characterizing the population structure of R. zeae. Population structure of Rhizoctonia zeae is being characterized (see technical report), which will enable a deeper insight into the biology and mode of spread of this pathogen. This information will be used to improve control strategies for this pathogen.

***5. List where the Project Research Results/Findings were Publicized:***

One Radio Interview:
1. Dr. Adesemoye had an interview on Kody Radio AM 1240 on June 21, 2017 during which the impact of soilborne pathogens on soybean, including Rhizoctonia and the integrated management of the pathogens were discussed.

Six Oral Presentations:
1. Kodati, S., Eskelson, M. J., and Adesemoye, A. O. 2017. Cross-pathogenicity of Rhizoctonia spp. isolated from multiple hosts to corn, soybean, and wheat. Annual Meeting of the North Central Region of the American Phytopathological Society, which held at Champaign, IL. June 14-16, 2017.
2. Adesemoye, A. O. presented a seminar in Lincoln, NE on September 29, 2017 titled: "Harnessing components of the root microbiome for integrated management of soilborne plant diseases" and findings from this study was part of the discussion. The seminar was part of the UNL Department of Agronomy and Horticulture fall seminar series, which was well publicized.
3. Kodati, S. and Adesemoye, A.O. 2018. Diversity and pathogenicity of Waitea circinata on row crops. ICPP: Rhizoctonia at crossroads: research advances and challenges, Boston, MA. Invited presentation.
4. Gambhir, N., Kodati, S., Adesemoye, A.O., and Everhart, S.E. 2018. Fungicide sensitivity of Rhizoctonia zeae from soybean and corn in Nebraska. ICPP: Rhizoctonia at crossroads: research advances and challenges, Boston, MA. Invited presentation.
5. Presentation during 2018 Nebraska Crop Management Conference at the Younes Conference Center, Kearney, NE on January 24 and 25, 2018.
6. An update on the project presented during the 2018 Crop Production Clinics at the Sandhills Convention Center, North Platte, NE on January 11, 2018.

Four Poster Presentations:
1. Kodati, S., Gambhir, N., Everhart, S., and Adesemoye, A. O. (2017). Prevalence and pathogenicity of Rhizoctonia spp. from soybean in Nebraska. A poster presentation during the American Phytopathological Society (APS) Annual meeting (poster #546-P), which held at San Antonio, Texas. August 5-9, 2017.
2. Kodati, S. and Adesemoye, A. O. 2018. Emerging understanding of the pathogenesis of Rhizoctonia zeae in row crops. ICPP-APS Joint Conference holding August 1 to 5 in Boston, MA.
3. Gambhir, N., Kodati, S., Adesemoye, A.O., and Everhart, S.E. 2018. Fungicide sensitivity of Rhizoctonia spp. isolated from soybean fields in Nebraska. Poster at ICPP Meeting in Boston, MA.
4. Gambhir, N., Kodati, S., Adesemoye, A.O., and Everhart, S.E. 2019. Fungicide sensitivity and population structure of Rhizoctonia zeae isolated from soybean and corn in the North Central U.S. Poster at the APS Annual Meeting held in Cleveland, OH.

Two CropWatch Articles:
1. Adesemoye, A. O. 2018. Root and Soilborne Diseases Update. CropWatch July 2, 2018.
2. Adesemoye, A. O. 2018. Soilborne and early seedling pathogens and delayed planting in corn and soybean. CropWatch May 3, 2018.

Two SoybeaNebraska Publications:
1. Gambhir, N., Everhart, S., Kodati, S., and Adesemoye, A. 2018. Fungicide Resistance: Risk and Management. SoybeaNebraska Mag., Spring 2018, Page 22.
2. Kodati, S., Adesemoye, A.O., Gambhir, N., and Everhart, S. 2018. Rhizoctonia Diseases in Soybean. SoybeaNebraska Magazine, Spring 2018, Page 23.

Ph.D. Student Dissertation:
1. Kodati, S. 2019. Diversity and Pathogenicity of Rhizoctonia spp. from Different Host Plants in Nebraska. University of Nebraska-Lincoln, Ph.D. Dissertation. ProQuest, in press.

Note: The above boxes will automatically accomodate for your text inputs; HOWEVER, the Final Report comprised of the above listed items must be kept to THREE PAGES. A Technical Report of no more than TEN PAGES (preferably fewer) can be appended to this report.

**Submit both reports as a single PDF with this file name format:** #XXX > FINAL > Project Title > PI last name

Please email this completed form to the Agriculture Research Division (jmonaghan2@unl.edu) based on the reporting schedule given to you. If you have any questions, please call the ARD at 2-2045 or Victor Bohuslavsky at the Nebraska Soybean Board Office at (402) 432-5720.

*Technical Report*

**Project Title:** Fungicide resistance in *Rhizoctonia solani* and implications for soybean fields in Nebraska

**Lead PI:** Sydney Everhart, Department of Plant Pathology, University of Nebraska-Lincoln
**Project Dates:** Oct. 1, 2015 – Sept. 30, 2019

## Introduction

Seedling diseases of soybean are estimated to cause losses of up to 1.35 million bushels in Nebraska [1]. Among other pathogens, *Rhizoctonia* spp. are causal agents. Species and sub-species, called Anastomosis Groups (AGs), of *Rhizoctonia* differ in their host specificity and virulence [5]. Information about the species and AG of *Rhizoctonia* in a region affects disease management recommendations. In Nebraska, *Rhizoctonia* spp. from soybean are uncharacterized, while such information is available for potato [2], dry bean [3], and sugar beet [4].

Fungicide seed treatments are recommended to protect soybean from seedling diseases. Such fungicides belong to four chemical classes: succinate dehydrogenase inhibitors (SDHI), quinone outside inhibitors (QoI), demethylation inhibitors (DMI), and phenylpyrroles (PP) [6]. Fungicide-resistant pathogens are an increasing threat to fungicide efficacy and can lead to economic losses if not detected. Thus, it is important to know if current seed treatments are effective against *Rhizoctonia* spp. that cause seedling diseases in Nebraska.

Factors related to emergence of fungicide resistance in field populations include evolutionary potential of the pathogen population, reproductive systems, and dispersal modes/mechanisms. This information can be obtained by studying the population structure of the pathogen. The structure of *R. solani* populations on rice, potato, tobacco and soybean, have been characterized previously, but no such studies have been performed in Nebraska. All previous studies characterized only AG-1 IA and AG-3 [7,8,9], which might not be the prevalent anastomosis groups in Nebraska.

**The objectives of this research project were to:**

1. Perform a comprehensive survey of soybean fields for *R. solani*;
2. Determine fungicide sensitivity of each isolate to selected commercial fungicides;
3. Identify the most prevalent anastomosis groups (within-species subgroups);
4. Develop and apply genetic markers to characterize the structure of populations within the two most prevalent *R. solani* subgroups.

## Objective 1: Perform a comprehensive survey of soybean fields for *R. solani*

Sample collection: An average of 18 soybean fields per year were in 2015, 2016, and 2017 and a grand total of 957 soil and plant samples were collected in a total of 54 field visits. From each field, soil samples were collected by walking a 'W' or 'Z' pattern transect and any symptomatic plants (with reddish-brown lesions on hypocotyl) were also collected. Samples were stored at 4 °C until processed. We also optimized laboratory methods for isolation and growth of Rhizoctonia, which will enable future studies to use standardized approaches to study this pathogen. These methods are described in more detail in *Sample processing*.

Evaluation of isolation methods: Symptomatic hypocotyl pieces were washed with distilled water and surface sterilized with 70% ethanol and placed on RM3 medium. Soil samples were processed using three methods: lab-based and greenhouse-based sugar beet seed baiting, organic debris floatation, and the toothpick method.

- *Lab-based sugar beet seed bait method:* Sugar beet seeds (*Beta vulgaris* L.) were used, as these are a known bait for *Rhizoctonia solani* [10]. Soil-samples were air-dried for two days. A 50mL sterile Falcon tube was half-filled with the soil sample and a cheese cloth sack filled with 10 twice-sterilized seeds was kept on the soil and covered with some additional soil to ensure that seeds made contact with the

soil. Added were 5mL of sterile distilled water to moisten sack. Cap was loosely closed and kept in dark for 3 days at 25 °C. After 3 days, seeds were removed from soil, cleaned with distilled water for one minute, followed by washing with 70% ethanol for 40 seconds and a final wash with distilled water for one minute. Seeds were air dried for two hours and transferred on to different semi-selective media

- *Greenhouse-based sugar beet seedling bait method:* Soil samples were put in 4-inch diameter clay pots in a saucer at room temperature and sugar beet seeds from a non-treated and root-rot-susceptible cultivar were sowed. Seedlings were monitored for symptoms until four weeks, and diseased plants were plated on different semi-selective media.
- *Organic debris floatation method:* Soil was placed in a container and distilled water added until organic debris floated. After 3-4 minutes of settling, floating organic debris were picked and placed on semi-selective media.
- *Toothpick method:* Soil samples were put in 4-inch diameter clay pots and four sterilized toothpicks were placed in each pot. The experiment was conducted at 23 ± 2°C. After two days, toothpicks were placed on semi-selective media.

Evaluation of growth media: Different semi-selective media were evaluated for isolating *Rhizoctonia* spp. including the modified Ko and Hora medium (Table 1), TS Medium (Table 2), and RM3 medium (Table 3).

**Table 1.** Ingredients for modified Ko and Hora medium*

| Components | Amount |
|---|---|
| $K_2HPO_4$ | 1 g |
| $MgSO_4.7H_2O$ | 0.5 g |
| KCl | 0.5 g |
| $FeSO_4.7H_2O$ | 10 mg |
| $NaNO_2$ | 0.2 g |
| Chloramphenicol | 50 mg |
| Metalaxyl | 63.3 mg |
| Prochloraz | 5 mg |
| Gallic acid | 0.4 g |
| Streptomycin sulfate | 50 mg |
| Agar | 20 g |
| Distilled water | 1000 ml |

*Hennis *et al.* 1978, Ko and Hora 1971, Castro *et al.* 1988

**Table 2.** Ingredients for TS medium[a]

| Components | Amount |
|---|---|
| Metalaxyl | 0.09 g |
| Potassium phosphite | 309.15 mg |
| Rifampicin solution | 100 µl (in 10 mg/ml dimethyl sulfoxide) |
| Ampicillin salt | 0.25 g |
| Moorehead agar | 7 g |
| Thiophanate-methyl | 0.494 mg to 2.47 mg |
| Distilled water | 1000 ml |

[a]Spurlock 2013

**Table 3.** Ingredients for RM3 medium

| Components | Amount |
|---|---|
| Streptomycin sulfate | 100 mg |
| Penicillin-G-sodium salt | 100 mg |
| 1N NaOH | 800µL |
| Agar | 18 g |
| Distilled water | 1000 ml |

Results from Obj. 1: Results showed the most effective method was the toothpick method and was used for further isolations. We also found that the most effective medium was the RM3 medium and was used for further isolations. We processed a grand total of 957 soil and plant samples. From these, 115 *Rhizoctonia spp.* were isolated and identified to species / sub-species using gene sequencing. Gene sequencing determined there were 80 isolates of *Rhizoctonia* zeae, 26 of *R. solani* AG-4, two of *R. solani* AG-3, one of *R. solani* AG-2, four of *R. solani* AG 1-IB, two of AG-B. Isolates used from this objective were characterized further in Objs. 2,3, and 4 below.

## Objective 2: Determine fungicide sensitivity of each isolate to selected fungicides

Fungicide sensitivity was determined for 51 *R. zeae* isolates from our collection and six collected in 2012 from Illinois [5]. Four recommended fungicide seed treatments were tested: azoxystrobin (QoI), fludioxonil (PP), prothioconazole (DMI), and sedaxane (SDHI). When possible, fungicide sensitivity was determined using an *in vitro* approach in the lab, however, for one fungicide (azoxystrobin), greenhouse assays were required.

### *In vitro* Fungicide Sensitivity

Preparation of fungicide amended media: Appropriate volumes of stock solution were added to PDA to produce final concentrations (Table 4). For azoxystrobin, 100 ppm salicylhydroxamic acid (SHAM) was also added to media to block the alternative oxidase pathway. Non-amended PDA served as control. SHAM was added in the controls for azoxystrobin assay.

**Table 4.** Concentrations of fungicides used to estimate $EC_{50}$ and the median and range of $EC_{50}$ across all isolates for each fungicide in *in vitro* studies.

| Fungicide | Final Concentrations (ppm) | Median $EC_{50}$ (ppm) | Range of $EC_{50}$ (ppm) |
|---|---|---|---|
| Azoxystrobin | 0, 50, 100 | >100 | >100 |
| Fludioxonil | 0, 0.01, 0.05, 0.1, 0.25, 0.5 | 0.096 | 0.029 - 0.386 |
| Prothioconazole | 0, 0.05, 0.1, 0.5, 1, 10 | 0.173 | 0.075 - 4.132 |
| Sedaxane | 0, 0.001, 0.005, 0.01, 0.1, 1 | 0.079 | 0.053 – 0.220 |

Data generation and collection: Sclerotia were revived on water agar (1.5%) at 25°C in dark. After two days, a mycelial plug was excised from the actively growing edge of the colony, transferred to PDA, and incubated at 25°C in dark. After two days, a plug was excised from the actively growing culture, inverted, and placed onto fungicide-amended PDA plates. Three technical replicates were made for each isolate x fungicide-concentration. After incubating at 25°C in dark for 48 h, mycelial diameter was measured in two perpendicular directions. The experiment was replicated.

Data analysis: Dose-response curve was fit to data for each isolate using log-logistic regression (LL.3 model) with the ezec package [11] in 'R', which is a wrapper of the drc package [12]. Relative Effective Concentration of 50% inhibition ($EC_{50}$) was estimated for each isolate.

Results of *In vitro* assays: Isolates were sensitive to all fungicides except azoxystrobin (Table 4). ANOVA was performed to check if $EC_{50}$ of isolates differed according to the year or crop of collection. Isolates collected from different years did not have any significant difference ($P > 0.05$) for all fungicides. Isolates collected from corn had significantly lower $EC_{50}$ for prothioconazole and sedaxane ($P < 0.05$).

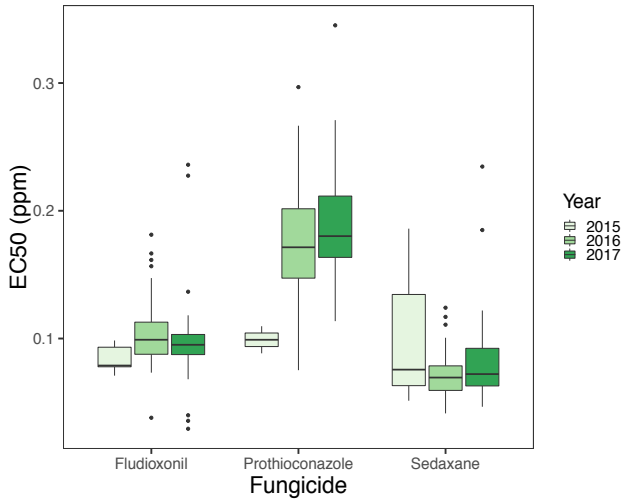


**Fig. 1** $EC_{50}$ did not differ significantly ($P > 0.05$) among isolates by year of collection

Until now, four to five doses were used to determine $EC_{50}$ of *R. zeae* isolates to fludioxonil, prothioconazole, and sedaxane fungicides. To make further screening cost and time effective, one discriminatory dose was identified for each fungicide. This dose was selected such that it could predict the $EC_{50}$ of each isolate. A linear regression model between log $EC_{50}$ and relative growth at each dose of each fungicide was generated. Dose at which the model showed best correlation coefficient ($R^2$) was chosen as the discriminatory dose. The model was validated using another dataset. Discriminatory dose for all fungicides was identified as 0.1 ppm.

Trials were conducted to accurately determine $EC_{50}$ for Azoxystrobin. Growth media were amended with various combinations of salicylhydroxamic acid and propyl gallate. These chemicals are known to aid in determining sensitivity to azoxystrobin in various fungi. However, even with the addition of these chemicals, the average $EC_{50}$ was more than 100 ppm and the exact $EC_{50}$ could not be estimated. A third chemical, octyl gallate, was also tested but since the results were still negative, fungicide sensitivity was determined *in planta*.

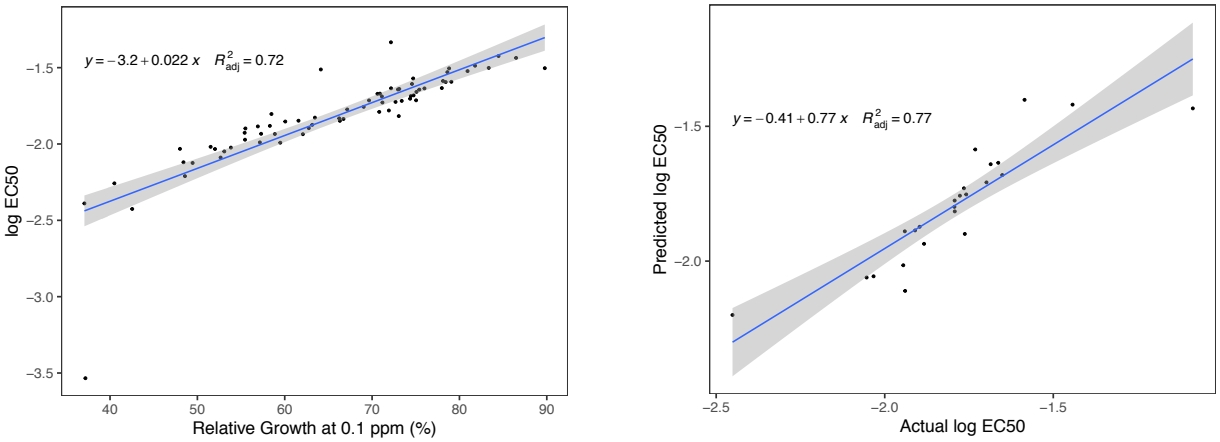


**Fig. 2** A linear regression model between log $EC_{50}$ and relative growth at each dose of prothioconazole was generated. Dose at which the model showed best correlation coefficient ($R^2$) was chosen as the discriminatory dose. The model was validated using another dataset. Discriminatory dose for all fungicides was identified as 0.1 ppm using the method described above.

### *In planta* Sensitivity to Azoxystrobin Fungicide

Since sensitivity to azoxystrobin could not be reliably determined *in vitro, in planta* sensitivity assays were conducted for two *R. zeae* isolates from Nebraska and one *R. zeae* isolate from Illinois. Soybean (Williams 82) seeds were treated with Dynasty (Syngenta) at a rate of 298.9 µl per kg seed. Plastic pots (8" diameter) were half-filled with 2:1 steam pasteurized sand: soil mixture, covered with 2 g inoculum, and then filled with a 5 cm layer of 2:1 steam pasteurized sand: soil mixture. Each pot was planted with 9 seeds. The experiment was set up as a randomized complete block design with four replications. The experiment was conducted in a growth chamber at a day/night temperature of 25°C/21°C and a 14 h photoperiod. After 18 days of planting, the plants were rated on a scale of 0-5, where a score of 0 meant no symptoms and a score of 5 meant that the plant was dead or with no roots. Based on this scale, a disease severity index was calculated. Dry weights of roots and shoots were also taken. The experiment was conducted twice. Azoxystrobin did not significantly change ($P > 0.05$) the disease incidence, disease severity, stand count, and total weight of the plants. This indicates that *R. zeae* may have inherent tolerance to this fungicide and that producers should use fungicide with other modes of action to control *R. zeae.*

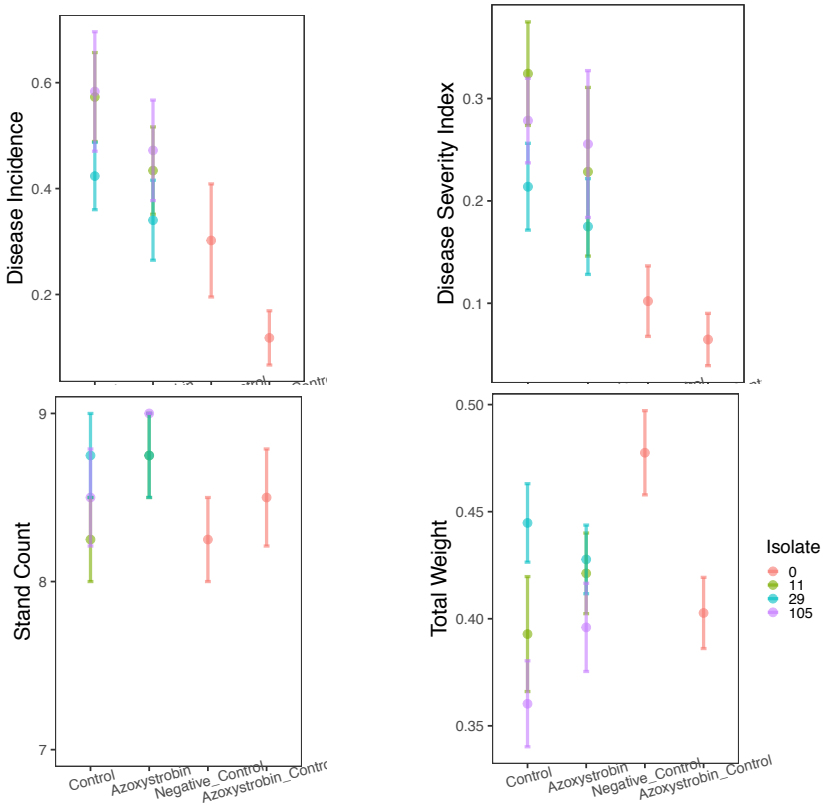


**Fig. 3** Mean and standard errors obtained in the first experiment for disease incidence, disease severity index, stand count, and total soybean plant weight in *in planta* assessment of azoxystrobin sensitivity of three *R. zeae* isolates (11, 29, and 105). Isolate 0 represents control.

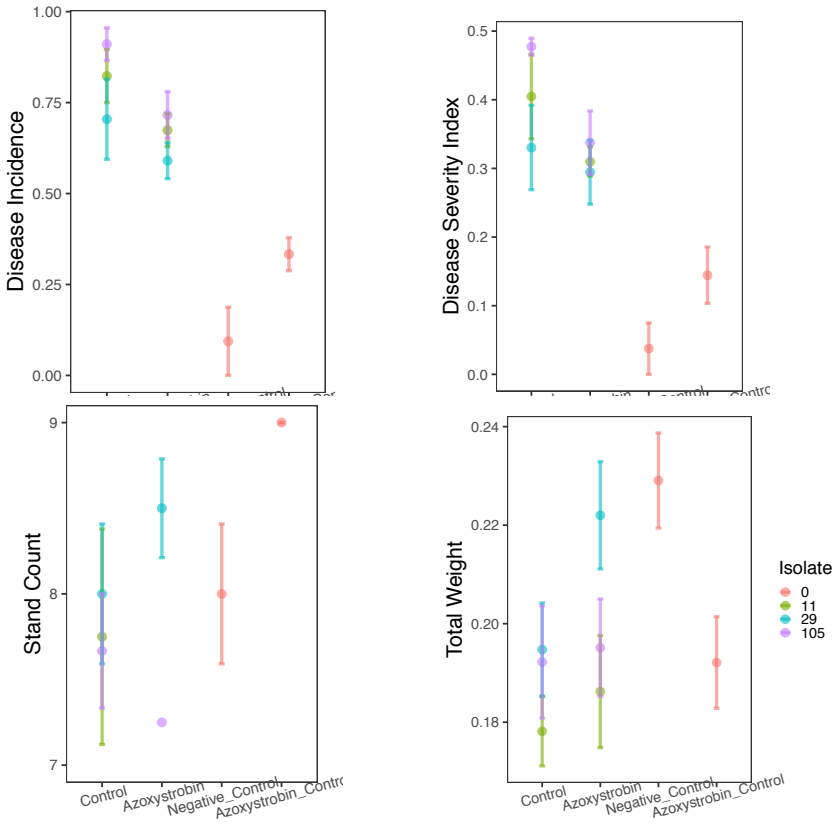


**Fig. 4** Mean and standard errors obtained in the second experiment for disease incidence, disease severity index, stand count, and total soybean plant weight in *in planta* assessment of azoxystrobin sensitivity of three *R. zeae* isolates (11, 29, and 105). Isolate 0 represents control.

## Objective 3: Identify the most prevalent anastomosis groups (within-species subgroups)

### Identification the most prevalent groups of *Rhizoctonia* spp.

A total of 115 isolates were collected during this survey from soybean fields. Preliminary identification was done based on morphological features like right angle branching pattern growth of hyphae on the media. DNA was extracted from pure cultures of 80 *Rhizoctonia* spp. isolates using a buffer extraction method. Polymerase chain reaction (PCR) was conducted using internal transcribed spacer (ITS) and β-tubulin (BT) primers to amplify the ITS1, ITS2, and 5.8S regions of ribosomal DNA, and β-tubulin gene regions respectively. Amplicons were purified and sequenced. These sequences were used to perform Nucleotide Basic Local Alignment Search Tool (BLASTn) searches and compared with sequences with the National Center for Biotechnology Information (NCBI) database for the identification of isolates to their subgroups. ITS consensus sequences of 25 *R. zeae*, 17 AG-4, three AG-1 IB, two AG-3, two AG-B, and one AG-2 isolates were submitted, and BT sequences of these isolates will be submitted to GenBank. Among the total isolates from our collection, *R. zeae* and *R. solani* AG-4 are found most prevalent species and groups.

## Objective 4: Develop and apply genetic markers to characterize the structure of populations within the two most prevalent *R. solani* subgroups

Reduced-representation whole-genome sequence data (GBS) of 12 *R. zeae* were obtained from collaborators in an attempt to design candidate markers using existing data. Data were filtered and 2,161 loci were identified as potential candidates for developing Single Nucleotide Polymorphism (SNP) primers. However, further analysis showed that the length of the DNA sequence available in the data was too short for designing primers. Hence, a new approach was used to design Simple Sequence Repeat (SSR) primers.

DNA was extracted from five *R. zeae* isolates and sent for whole genome sequencing. A total of 1,594 candidate SSR loci were identified. We designed and synthesized 24 SSR primers, which showed polymorphisms *in silico*. DNA was extracted from 80 *R. zeae* isolates. A touchdown Polymerase Chain Reaction (PCR) assay was performed on seven of these isolates to validate amplification and polymorphism of the designed primers. Out of 24 primers, six primers showed consistent amplification and polymorphism. One of the loci was a pentanucleotide repeat and the remaining five loci were trinucleotide repeats. The number of alleles for these loci ranged from four to seven. The average gene diversity and evenness were 0.74 and 0.78, respectively. These statistics showed that the six markers were of good quality. However, further analysis showed that five of these loci did not amplify in most of the isolates. Nineteen more primers were designed, which will be used in further PCR assays. This project will be completed as part of Nikita Gambhir's dissertation; she is targeting 2020 for graduation.

### References

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