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Case Report

Interaction of Host Genotype, Medium of Inoculum, and Pathogen Species on Pathogenicity of *Pythium* Disease on Soybean

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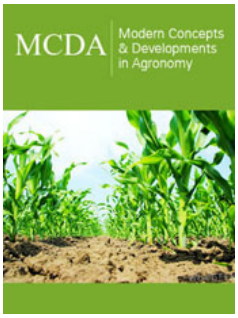

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Abstract

Soybean (*Glycine max* (L.) Merr.) is an important crop for edible oil and protein in the United States and the world. However, soybean yield productions have been challenged by diseases caused by soil-borne pathogens such as *Pythium*. The purpose of this case study was to identify the interactions among soybean genotypes, medium of inoculum, and pathogen species on the pathogenicity of *Pythium* disease on soybean. Three-way ANOVA indicated that the interaction of host genotype, inoculum medium and pathogen species significantly affected the severity of *Pythium* disease on soybean. There were also significant interactions between inoculum medium and pathogen species. Marginal analysis for inoculum medium indicated that rice may be a better medium used for inoculation for *Pythium* than millet as it induces higher pathogenicity on soybean plants. Marginal analysis for *Pythium* species indicated that *P. oopapillum* was the most aggressive on 6 soybean genotypes evaluated by RR and on 4 genotypes by RW; *P. irregulare* was the most aggressive on 2 soybean genotypes evaluated by RR, and on 4 genotypes by RW; and *P. sylvaticum* was the most aggressive on one soybean genotype by both RR or RW.

Keywords: Soybean; *Pythium* root rot; Medium

Abbreviations: ANOVA: Analysis of variance; RR: Root Rot Rating; RW: Ratio of Plant Fresh Weight

Introduction

Soybean (*Glycine max* (L.) Merr) is one of the most important crops for edible oil and proteins in the United States. However, about 11% of soybean production is suppressed by soybean diseases including seedling disease [1,2]. In the U.S. and Ontario, Canada, seedling diseases caused a total yield loss of 241 million bushels from 2010 to 2014, being the second most destructive soybean diseases [1]. One of the most important pathogens causing soybean seedling diseases is *Pythium*, a genus of soil-borne, oomycete pathogens typically favored by cool, wet conditions and early planting, causing seed decay, root rot, and damping off [3]. More than 20 *Pythium* species have been identified with pathogenicity on soybean so far, such as *P. ultimum*, *P. aphanidermatum*, *P. debaryanum*, *P. irregulare*, *P. myriotylum*, *P. torulosum* and *P. vexans*, in Ohio, Iowa, Florida, Arkansas, Illinois, and North Dakota [4-12]. All the *Pythium* species cause similar symptoms, including soft and rotted seeds before germination, pre- or post-emergence damping-off, and hypocotyl or root discoloration [13,14]. Crop rotation is a traditional way to manage *Pythium* diseases [15], however, because *Pythium* has multiple hosts such as corn and wheat, crop rotation is considered less effective [13]. Another widely used method is the treatment of seeds with fungicide, such as metalaxyl, mefenoxam and some newer fungicides [16-18].

However, the continued use of these fungicides may lead to reduced efficacy due to increased insensitivity of the *Pythium* population. In addition, fungicide has been more and more widely considered as less desirable for environment and health concerns [19]. The most cost-effective and environmental-friendly method to control disease is the deployment of resistant soybean varieties. To evaluate the soybean seedlings at early stage, greenhouse essay has been developed and used in several studies and rice and millet are the most used

medium for inoculum [20-29]. However, it has not been clear yet about which medium is more suitable to evaluate the pathogenicity of Pythium on soybean, and moreover, it is still unknown whether there's any interactions between soybean genotypes, the inoculum medium, and different *Pythium* species, which would strongly impact our strategy of evaluating *Pythium* diseases. Therefore, this case study was designed to

1) explore the interactions of soybean genotype, inoculum medium, and *Pythium* species;

2) identify a more suitable medium for *Pythium* disease evaluation; and

3) Compare the pathogenicity of different *Pythium* species

Case Presentation

A total of 9 Michigan State University improved soybean lines were used in this study. For the pathogen, three *Pythium* isolates were used including CMISO2 5-14, MSIO 8-23, and CMISO2 2-30, which were classified as *P. irregulare*, *P. oopapillum*, and *P. sylvaticum*, respectively [3]. All isolates were maintained on slants of Potato Carrot Agar (PCA) at 15 °C in glass tube. A modified protocol from Howell [30] was used to prepare inoculum (Figure 1A). Two cultural media, millet and white rice, were used. 625g millet seeds and 425g white rice (parboiled) grains were each mixed with 400mL ddH_2O in an autoclave-safe plastic bag and autoclaved at 121 °C for 4 hours. When bags cooled down to room temperature, one full plate of a 4-day-old isolate cultured at CMA medium was transferred to the millet or rice bag aseptically. After incubation at room temperature for 12 days, the inoculum was ready to use (Figure 1B). The disease was evaluated at the Michigan State University greenhouse, with temperature at 20-22 °C and humidity at 20%. All soybean varieties were evaluated in a randomized incomplete block design with 12 seeds evaluated for each replicate. Three replicates were performed for inoculation group, and one replicate was used as control. To start the assay, 6-cell seed starting trays were first fulfilled with medium size vermiculite and soaked in water until the vermiculite was fully saturated. Then two 2cm-depth holes were made in each cell and approximately 2g inoculum and un-inoculated medium (or 6-8 rice grains or 15-20 millet grains) was placed at the bottom of each hole for inoculation. A single seed was then planted on the top of the inoculum in each hole and covered with vermiculite (Figure 1C). After planting, seed starting trays were transferred to the greenhouse benches which had been covered with waterproof plastic. Benches were watered until the water height reached the height of inoculum. After that, benches were watered every other day to maintain the water saturation environment until two days before measurement. Data were collected when the control group reached V2 stage, or 14 days after inoculation (Figure 1D). Root score and whole plant weight were measured. To rate root score, roots were removed from the pots, gently washed and cleaned with running tap water, and wrapped in a towel to absorb excess water.

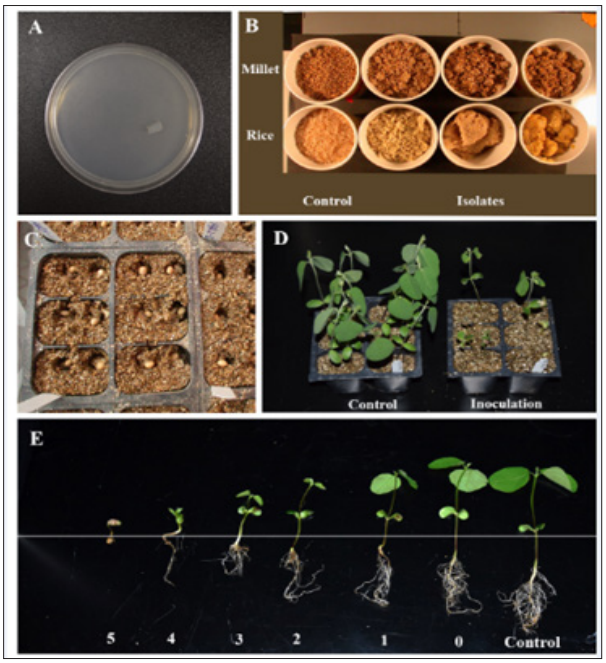

Figure 1: Methods used to evaluate soybean partial resistance to *Pythium irregulare*.

A: Growth of *P. irregulare* isolate (CMISO2 5-14) on the surface of corn meal agar (CMA) on the fourth day. B: Pythium inoculums produced on millet (upper) and rice (lower) media for twelve days.

C: Planting of seeds and inoculum in half-inch deep holes.

D: Example of inoculated and control plants 14 days post inoculation (dpi).

E: Response of soybean plants to *P. irregulare* 14 dpi. Numbers beneath each plant indicates the root rot (RR) scores with 5 for kill, and 0 for complete health.

Root rot score for each plant was rated on a 0-to-5 scale (Figure 1E), wherein, 0 represents a healthy root system with no symptoms of lesions or rot on the roots; 1 represents small lesions and discoloration on the roots, with approximately 1% to 20% of roots showing visible symptoms; 2 represents some lesions on roots and lateral roots with approximately 20 to 40% of roots rotted with visible symptoms of lateral roots reduction; 3 represents rot on lateral roots and visible symptoms of rot, with approximately 40 to 60% of the roots exhibiting visible symptoms; 4 represents both lateral roots and main tap roots having visible symptoms on roots and approximately 60 to 90% of the roots infected, roots and lateral roots reduced drastically, and 5 represents roots severely rotted or no germination with complete colonization on the seed. Whole plant weight was measured using a bulk of 12 plants for each replicate using an electronic balance (Scout Pro, SP 4001; Ohaus Corp, Pine Brook, NJ). To evaluate the disease, root rot rating (RR) and ratio of fresh plant weight (RW) were used. Root rot rating (RR) was calculated as ∑[5×(number of roots at 5) + 4×(No. of roots at 4) + 3×(No. of roots at 3) + 2×(No. of roots at 2) + 1×(No. of roots at 1) + 0×(No. of roots at 0)]/[5× (No. of control roots)]; Ratio of plant fresh weight (RW) was as [Fresh plant weight of inoculated plants / (No. of inoculated seeds] / [Fresh plant weight of control plants / (No. of control plants)]. The analysis of variance (ANOVA) for RR and RW were analyzed using the general linear model procedure (GLM) of SPSS 22 (SAS Institute Inc.) and the results were considered significant with $p<0.05$. Discrimination among the means was conducted using Fisher's least significant difference (LSD) procedure.

Results and Discussion

The three-way ANOVA results indicated that there were significant interactions among soybean genotypes, inoculum medium, and *Pythium* species for both RR and RW with p=0.006 and p=0.026, respectively (Table 1). Among the three factors, soybean genotype did not have significant interaction with inoculum medium for either RR or RW (p=0.071, and p=0.126, respectively). The interaction between soybean genotype and *Pythium* species was significant for RR, but not significant for RW (p=0.046, and p=0.611, respectively). However, the interaction between inoculum medium and *Pythium* species was very significant with $p<0.001$ for both RR and RW. These results indicated that inoculum medium is important for evaluation of soybean diseases caused by different *Pythium* species. To further evaluate the effect of inoculum medium on soybean diseases, we conducted the marginal analysis as shown in Figure 2. For each soybean genotype, the RR values obtained from rice was consistently larger than that from millet (Figure 2A). Averagely, the mean of RR for rice was 0.817, compared to that of millet which was 0.672. Because the higher value of the RR indicates higher disease pathogenicity, the inoculation method using rice as inoculum medium can therefore induce higher disease pressure to soybean plants. The results from RW was consistent with that from RR (Figure 2B). For RW, the lower value indicates higher disease pressure. Obviously, all the nine soybean genotypes were consistently more susceptible using rice than using millet as inoculum medium, with RW averagely 0.394 and 0.645 for rice and millet, respectively. Our results indicated that for future studies on *Pythium* diseases, rice may be a better choice than millet as inoculum medium to obtain higher disease pressures.

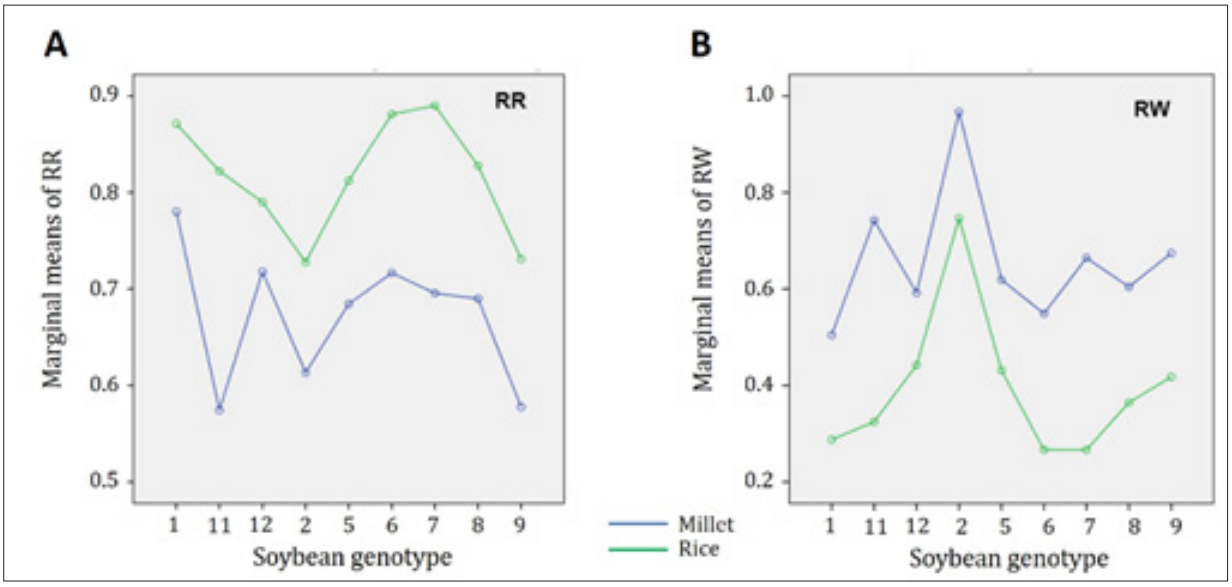

Figure 2: Comparison of inoculum medium on the pathogenicity of three *Pythium* species on nine soybean genotypes.

A: Evaluation of the root rot rating (RR) for millet (blue) and rice (green).

B: Evaluation of the ratio of plant weight (RW) for millet (blue) and rice (green).

Table 1: Three-way ANOVA for interaction of soybean genotype, inoculum medium, and pathogen species on the pathogenicity of *Pythium* diseases on soybean plants, evaluated by root rot rating (RR) and ratio of plant fresh weight (RW).

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	RR	2.582[a]	53	0.049	7.095	0
	RW	8.465[b]	53	0.16	6.112	0
Intercept	RR	89.825	1	89.825	13082.44	0
	RW	43.722	1	43.722	1673.101	0
Genotype	RR	0.502	8	0.063	9.136	0
	RW	2.164	8	0.27	10.349	0
Medium	RR	0.849	1	0.849	123.701	0
	RW	2.544	1	2.544	97.343	0
Isolate	RR	0.181	2	0.091	13.209	0
	RW	0.183	2	0.092	3.503	0.034
Genotype * Medium	RR	0.103	8	0.013	1.879	0.071
	RW	0.34	8	0.043	1.627	0.126
Genotype * Isolate	RR	0.193	16	0.012	1.76	0.046
	RW	0.361	16	0.023	0.864	0.611
Medium * Isolate	RR	0.498	2	0.249	36.23	0
	RW	2.073	2	1.037	39.667	0
Genotype * Medium * Isolate	RR	0.255	16	0.016	2.322	0.006
	RW	0.8	16	0.05	1.914	0.026
Error	RR	0.742	108	0.007		
	RW	2.822	108	0.026		
Total	RR	93.148	162			
	RW	55.009	162			
a. R Squared = .777 (Adjusted R Squared = .667)						
b. R Squared = .730 (Adjusted R Squared = .598)						

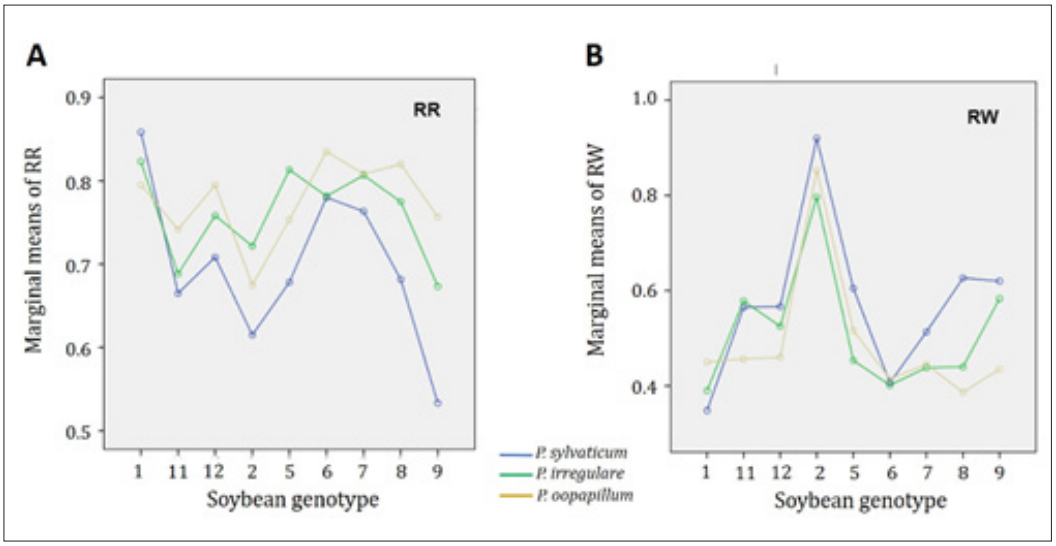

Figure 3: Comparison of the pathogenicity of three *Pythium* species on nine soybean genotypes.
A: Evaluation of the root rot rating (RR) for three *Pythium* species.
B: Evaluation of the ratio of plant weight (RW) for three *Pythium* species

Additionally, we also conducted the marginal analysis to compare the pathogenicity among the three isolates from the three *Pythium* species (Figure 3). The pathogenicity of three isolates was generally consistent on soybean genotypes using either RR or RW as evaluation method. For example, for soybean genotype1, CMISO2 2-30 (*P. sylvaticum*) was the most pathogenic as indicated by both RR and RW, followed by CMISO2 5-14 (*P. irregulare*) and MISO 8-23 (*P. oopapillum*), while for soybean genotype 9, the most aggressive isolate was the *P. oopapillum*. The *P. oopapillum* isolate was most aggressive on six soybean genotypes evaluated by RR (11,12,6,7,8 and 9) and on four soybean genotypes by RW (11,12,8 and 9). The *P. irregulare* isolate was most aggressive on two soybean genotypes (2 and 5) by RR, and on four genotypes (2,5,6, and 7) by RW. The *P. sylvaticum* isolate was the most aggressive only on soybean genotype 1.

The different response pattern of soybean genotypes to different species of *Pythium* suggested that soybean does not deploy a single genetic resistance mechanism against different *Pythium* species. This can be supported by the identification of different quantitative disease resistance loci (QDRL) for different types of *Pythium* species. For example, our previous study identified two QDRL on soybean chromosomes 11 and 20, respectively, for partial resistance to *P. irregulare* [23], while in our recent study for soybean resistance to *P. sylvaticum*, novel QDRLs were identified on chromosomes 10 and 18 (unpublished data). Nevertheless, some common genes may play critical roles in regulating soybean resistance to different *Pythium* species. For example, the QDRL on chromosome 20 was identified for both *P. irregulare* and *P. sylvaticum* (unpublished data).

Summary

This case study illustrated that soybean genotypes, inoculum media, and types of *Pythium* isolates, along with the interactions between inoculum media and types of *Pythium* isolates, and the interactions among the three factors, can significantly affect the soybean response to *Pythium* pathogen which was evaluated by RR and RW. Compared to millet medium, rice medium can induce heavier disease severity and may serve as a better choice for soybean disease resistance studies for *Pythium* root rot. In addition, this case study indicated that *P. oopapillum* was the most aggressive on 6 soybean genotypes evaluated by RR and on 4 genotypes by RW; *P. irregulare* was the most aggressive on 2 soybean genotypes evaluated by RR, and on 4 genotypes by RW; and *P. sylvaticum* was the most aggressive on one soybean genotype. Moreover, our results suggested that different mechanisms of resistance may be deployed by soybean plants although some common genes may play important roles for resistance to different *Pythium* species.

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