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Diversity of *Pythium* spp. associated with soybean damping-off, and management implications by using foliar fungicides as seed treatments

Shrishail S. Navi*, Tra Huynh, Chase G. Mayers and Xiao-Bing Yang

Abstract

Soybean (*Glycine max*) seedlings with symptoms of Pythium damping-off were collected in northeastern Iowa soybean fields and processed for isolation of the causal agents on both potato dextrose agar (PDA) and pimaricin-, ampicillin-, rifampicin-, and pentachloronitrobenzene (PARP)-containing media. Isolates were identified based on morphological characteristics, growth rates, along with sequence data for the nuclear rDNA ITS1–5.8S-ITS2 region (ITS barcode). Nine isolates were identified via NCBI BLASTn search of sequences available in GenBank: one isolate of *Pythium orthogonon*; three isolates of *P. inflatum*; two isolates of *P. ultimum* var. *ultimum*; one isolate of *P. torulosum*; and two isolates of *P. ultimum* var. *ultimum* or *P. ultimum* var. *sporangiferum*. Pathogenicity of all the nine isolates, along with a positive control (*P. irregulare*), was tested in greenhouse conditions on soybean variety Pioneer 22T61R. Soybean seeds were planted in potting mixture inoculated with *Pythium* inoculum fermented on sterilized proso millet grains. The *Pythium* spp. were subsequently re-isolated from symptomatic plants. Average incidence of Pythium damping-off across isolates was 27.4% but varied among isolates, ranging from 1.2 to 79.8%. Among the *Pythium* spp. collected in this single location, the most aggressive isolate was selected to test the efficacy of seed treatments using foliar fungicides in artificially-inoculated field conditions. Out of the eight tested foliar fungicides, six of them significantly suppressed damping-off compared with the untreated control. The average yield advantage of foliar fungicides as seed treatments was 0.23 mt (metric ton)/ha (ranged from 0.15 to 0.31 mt/ha) over the untreated control, with a corresponding economic advantage of $90.69 (range $60.5 to $123.9/ha) based on soybean price at $397/mt as of September 30, 2017. Our findings suggest a potential for using foliar fungicides as seed treatments to control Pythium damping-off, and provide an alternative solution for managing resistance to metalaxyl/mefenoxam seed treatments in soybean production.

Keywords: Pythium damping-off, Soybean, Foliar fungicides, Seed treatments

Background

Worldwide more than 200 pathogens affect soybean (*Glycine max* [L.] Merr.), of which at least 35 are considered economically important (Hartman 2015). In Iowa these include several species in the genus *Pythium* (Oomycota) that cause damping-off and root rot symptoms in soybean seedlings. *Pythium* spp., which have very wide host range including grasses and a wide variety of dicotyledonous plants (Waterhouse and Waterston 1964), cause problems in stand establishment, reduce seedling emergence, kill emerged seedlings, and reduce plant vigor (Kirkpatrick et al. 2006; Broders et al. 2009). One of the biggest threats for US soybean growers is the rapid speed at which *Pythium* attacks soybean seed; the dormant propagules (oospores) of *Pythium* spp. germinate in response to seed and root exudates and can infect seeds within 90 min of planting (Stranghellini and Hancock 1971). Infection by *Pythium* spp. leads to seed rot and the premature weakening and death of developing seedlings, referred to as damping-off (Kirkpatrick et al. 2006). Cooler soil temperatures and

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higher soil moisture content promote infection (Brown and Kennedy 1966; Biesbrock and FFJr 1970a, b; Yang 2002; Matthiesen et al. 2016). Other important soil-borne pathogens that cause soybean seedling diseases include species of the oomycete genus *Phytophthora* (Erwin and Ribeireo 1996), as well as the fungal genera *Rhizoctonia* (Yang 1999), and *Fusarium* (Díaz Arias et al. 2013).

Between 1996 and 2009, totaled across 28 US states (Wrather and Koenning 2009; Koenning and Wrather 2010), soybean seedling diseases caused an average annual yield loss of 32.9 million bushels (0.90 million metric tons, mmt), ranging from 12.3 million bushels (0.34 mmt) in 1999 to 55.5 million bushels (1.51 mmt) in 2009. This represents significant economic losses due to soybean seedling diseases, ranging from $67.60 million in 1999 to $779.92 million in 2008, based on soybean prices at $201.67/mt and $522.83/mt, respectively (https://ycharts.com/indicators/us_soybean_price_world_bank).

The use of fungicide seed treatments in soybean increased from 8% of plantings in 1996 and 30% in 2008 (Munkvold 2009), to 60%–70% in 2014 (USB 2014), corresponding with global growth of the seed treatment market for soybean and other crops (Anon 2017; Wellesley 2013). Increased cost of seeds and early planting in spring may be a driving force for such changes. Fungicide seed treatments are especially effective in preventing or reducing damage from seed- or soil-borne pathogens that cause seed decay, seedling blights, and root rots (Sweets 2006), and thus result in a significant yield increase in fields with high seedling disease risk (Yang 2009). In optimum conditions, seed treatments can provide significant return on value; for example, Poag et al. (2005) determined that seed treatments costing less than $8.65/ha averaged a return of $43.71/ha due to protection against soil-borne and seed-borne pathogens in Arkansas. However, mixed results have been observed from the use of seed treatments in different environments and under different levels of pathogen pressure (Bradley et al. 2001; Dorrance and McClure 2001; Dorrance et al. 2003a; Bradley 2008). Most available fungicides are labeled for use against specific pathogenic fungi (e.g. *Rhizoctonia*, *Fusarium*, and *Macrophomina*), but some (i.e. oomicides) are intended for use against oomycetes (e.g. *Phytophthora* and *Pythium*) (Sweets 2006). Bradley (2010) suggested combining fungicides effective against oomycetes (mefenoxam or metalaxyl) with at least one other fungicide (e.g. fludioxonil, trifloxystrobin, pyraclostrobin, or ipconazole) to provide additional control of *Fusarium*, *Rhizoctonia,* or other fungal pathogens.

A diversity of *Pythium* spp. have been associated with diseases of soybean and corn seedlings in Iowa (Robertson 2012, Robertson et al. 2013), and a diverse array of oomycete species cause problems in soybean across North America (Rojas et al. 2017a, 2017b). These include additional pathogenic species of *Pythium* which have not yet been reported in Iowa, but may be present and were missed in the small number of previous surveys. In Iowa, as well as elsewhere in the North Central Region, the protection of seedlings from Phytophthora/Pythium damping-off and Rhizoctonia/Fusarium root rot mainly comes in the form of seed treatments (chemical and biological) specifically formulated for application to seeds (Bradley 2008). Interestingly, Powelson and Inglis (1999) identified a potential alternate use of foliar fungicides as seed treatments in potato. Considering the potentially incomplete understanding of the diversity of *Pythium* spp. present in Iowa, potentially significant losses due to the seedling disease they cause, and a need for alternative management strategies, we set three broad objectives in this work: (a) isolate and characterize a diversity of *Pythium* spp. associated with soybean seedlings in Iowa; (b) test the pathogenicity of representative isolates; and (c) field test fungicide seed treatments with Quinone outside inhibitors (QoI), Demethylation inhibitors (DMI) and Succinate dehydrogenase inhibitors (SDHI), against the most aggressive available isolate from the pathogenicity test.

Results

Soybean seedlings display Pythium damping-off symptoms in the field trial

Of the various planting dates, the June 8th plantings of varieties Pioneer 22T69R and Pioneer 25T51R showed symptoms of damping-off (Fig. 1), whereas Pioneer 92Y75 for the most part did not show damping-off. The three varieties germinated and emerged normally on

Fig. 1 Field symptoms of *Pythium* damping-off observed during June 2016 at Northeast Research and Demonstration Farm, Nashua, Iowa. Damped-off soybean seedlings are seen between two healthy seedlings and an inset is close up image of damping-off

June 13th, but after six days of rain starting on June 9 (15.2 cm), seedlings in the lower portion of the replications on the downhill side of the field began to show symptoms of damping-off.

Pythium isolates collected in Iowa comprise several different species or subspecies

Out of 16 isolates that produced oospores and sporangia (Fig. 2), and displayed culture morphology of *Pythium* spp., nine were selected for ITS barcoding due to differences in culture morphology. ITS sequences obtained from these isolates matched *Pythium* sequences in GenBank: "C1–2" had 99% (855/864 bases) identity with *Pythium orthogonon* HQ643723; isolates "D2–1", "D1–2", and "A2–1" had 100% (822/822, 814/814, and 823/823 bases, respectively) identity with *P. inflatum* AY598626; isolates "2–3" and "1–4" had 100% (876/876 and 870/870 bases, respectively) identity with *P. ultimum* var. *ultimum* KU210728; isolate "– 4" had 100% (826/826 bases) identity with *P. torulosum* AY598624; isolate "-?" had 100% identity with both *P. ultimum* var. *ultimum* KU211001 (662/662 bases) and *P. ultimum* var. *sporangiferum* KT429653 (659/659 bases); and isolate "-??" had 100% identity (886/886 bases) with the sequence obtained from isolate "-?"

Pythium isolates differ in colony morphology and growth rates

Colony growth rate in diameter (mm/day) (Table 1) and visual assessment of colony morphology (Fig. 3) indicated variability among the isolates. At an early stage of incubation (1–4 days after incubation), there were significant differences in radial growth among a few isolates, but such difference disappeared at 5 days post incubation (Table 1).

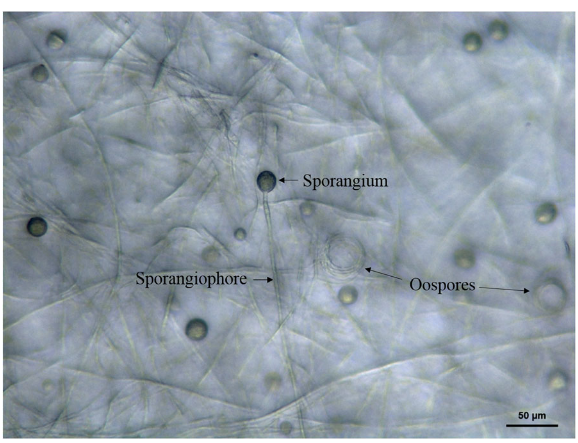

Fig. 2 Hyphae, sporangia, sporangiophore and oospores imaged with a Zeiss Axio Zoom V16 microscope using base illumination with relief contrast, dark-field and bright-field modes and epi-illumination with a Schott LED ring light to catch intact images of fruiting bodies of *Pythium irregulare*. This image was captured without opening culture plate at Bessey Microscope facility (currently, Microscopy and NanoImaging Facility), Iowa State University

Pythium isolates display pathogenicity diversity

Pathogenicity of all the nine isolates, along with a positive control (*P. irregulare*), was proven in greenhouse conditions on soybean variety Pioneer 22T61R. Seeds were planted in potting mixture mixed with individual *Pythium* isolates fermented on sterilized millet grains. Average Pythium damping-off incidence across isolates was 27.39%, ranging from 1.2% in isolate "A2–1" (*P. inflatum*) to 79.8% in isolate "2–3" (*P. ultimum* var. *ultimum*). Significantly ($P < 0.05$) highest incidence was observed in isolate 2–3 (Table 2). There was no significant difference in damping-off incidence among isolates "1–4" (58.3%), "?" (58.3%) and "??" (51.2%). Also, these isolates showed significantly higher incidence (%) compared with the positive control *P. irregulare* of 14.3% (Table 2). Other five isolates (D1–2, C1–2, – 4, D2–1, and A2–1) showed less than 5% incidence with no significant differences among these isolates and with the positive control (Table 2; Fig. 4).

Foliar fungicides effectively control soybean Pythium damping-off

An attempt was made to test the efficacy of QoI, DMI and SDHI fungicides in control of Pythium damping-off. Though, damping-off incidence in field test was not as high as that in control conditions. An average incidence in fungicide treated plots was 0.68% (ranged from 0.31% to 1.07%), compared to the untreated control of 1.21% (Table 3). Similarly, an average yield in fungicide treated plots was 5.28 mt/ha (ranged from 5.20 to 5.36 mt/ha) compared with 5.05 mt/ha in the untreated control (Table 3). Yields were significantly ($P < 0.05$) higher in plots treated with Aproach, Priaxor, Quadris, and Stratego YLD compared with the untreated control (Table 3). Also, yields were numerically higher in plots treated with Endura, Evito, and Headline compared with the control, but statistically no difference (Table 3). An average yield advantage across fungicide treated plots compared with that of untreated plots was 0.23 mt/ha (range from 0.15 mt/ha to 0.31 mt/ha), and corresponding economic advantage was USD 90.69/ha (range from $60.50/ha to 123.9/ha) based on soybean price $397/mt on September 30, 2017 (https://ycharts.com/indicators/us_soybean_price_world_bank).

Discussion

A diversity of *Pythium* spp. have been associated with soybean and corn seedling diseases across North America including Iowa (Robertson 2012; Robertson et al. 2013; Matthiesen et al. 2016; Rojas et al. 2017a,

Table 1 Radial growth rate (mm/day) of *Pythium* isolates recorded at 24 h interval for 5 days

Pythium isolates	Radial growth rate (mm/day) over 5 days of incubation*				
	1	2	3	4	5
P. inflatum	13.5 dc	31.0 dc	47.0b	62.5ba	63.8a
P. ultimum var. *ultimum*	43.5a	83.8a	85.0a	85.0a	85.0a
P. orthogonon	36.0b	72.0ba	85.0a	85.0a	85.0a
P. torulosum	0.0e	61.3b	85.0a	85.0a	85.0a
P. inflatum	0.0e	15.8d	46.8b	57.5ba	63.8a
P. inflatum	8.5d	26.0 dc	43.3b	62.0ba	85.0a
P. ultimum var. *ultimum*	11.0 dc	24.0 dc	35.5b	48.0b	62.3a
P. ultimum var. *ultimum* or *P. ultimum* var. *sporangiferum*	16.0c	33.3c	52.0b	71.3ba	77.5a
P. ultimum var. *ultimum* or *P. ultimum* var. *sporangiferum*	8.0d	25.3 dc	43.5b	61.0ba	85.0a

*Results are average of 4 replications. Means within column followed by the same letter(s) are not significantly different from each other at 5% level of significance ($P < 0.05$)

2017b). Earlier studies (Zhang et al. 1998; Zhang and Yang 2000) on soybean and corn rotation fields in Iowa found *Pythium* populations with four major species (*P. ultimum*, *P. torulosum*, *P. paroecandrum*, and *P. spinosum*), of which *P. torulosum* was the most frequently isolated species. In this study, nine *Pythium* isolates were obtained comprising five different species or subspecies, of which two (*P. orthogonon*, and *P. ultimum* var. *sporangiferum*) appear to be new reports for Iowa; the remaining species have been previously reported on either soybean or corn (Robertson et al. 2013; Matthiesen et al. 2016; Rojas et al. 2017a, 2017b). The fact that a sample of nine isolates yielded five species/subspecies agrees with previous studies and suggests that *Pythium* species composition is typically diverse within fields.

Management of Pythium damping-off mainly involves seed treatments with either chemical or biological products (Munkvold 2009; Coker et al. 2010), apart from cultural practices (Coker et al. 2001), and host-plant resistance (Dorrance et al. 2003b; Rosso and Rupe, 2005; Bates et al. 2008). Recently, Carmona et al. (2018) reported products of Spraytec fertilizers containing potassium and manganese phosphites as an alternative to fungicide seed treatments to control soybean damping-off caused by *P. aphanidermatum*, *P. irregulare* and *P. ultimum* in Argentina.

Although there are no known soybean varieties resistant to Pythium damping-off on the market, there are varieties differing in susceptibility to damping-off. Apart from soybean, *Pythium* spp. can infect a very wide range of hosts, including corn, and the oospores of *Pythium* are long lived in soil. Hence, the effectiveness of crop rotation (particularly corn in Iowa) in managing Pythium damping-off is limited.

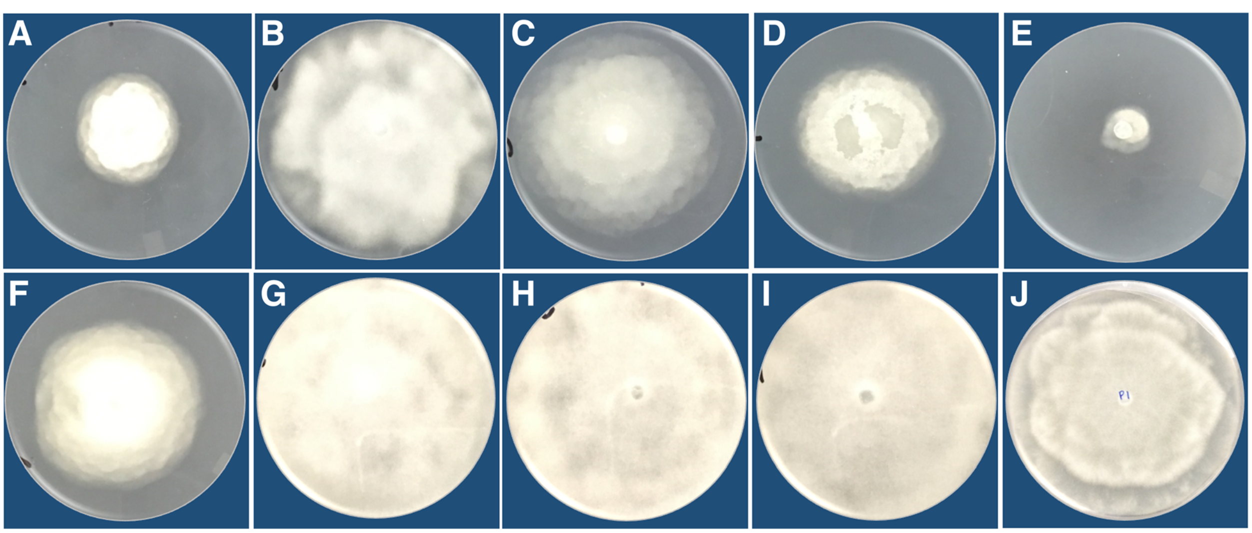

Fig. 3 Colony morphology of *Pythium* isolates at 3 days after incubation on PDA. *P. inflatum* (**a**, **e** and **f**), *P. ultimum* var. *ultimum* (**b**), *P. orthogonon* (**c**), *P. torulosum* (**d**), *P. ultimum* var. *ultimum* (**g**), *P. ultimum* var. *ultimum* or *P. ultimum* var. *sporangiferum* (**h** and **i**), and *P. irregulare* (**j**) incubated under a 12 h light/12 h dark cycle at 23 ± 1 °C

Table 2 Damping-off incidence (%) of *Pythium* isolates on soybean variety Pioneer 22T61RR in pathogenicity tests conducted in greenhouse[x]

Cups labeled	Isolate No.	Identity with GenBank (%)	GenBank Accession No.	*Pythium* spp.	Incidence (%)[y]
1	D1–2	100% (814/814 bases)	AY598626	*P. inflatum*	2.4c
2	1–4	100% (870/870 bases)	KU210728	*P. ultimum* var. *ultimum*	58.3b
3	C1–2	99% (855/864 bases)	HQ643723	*P. orthogonon*	3.6c
4	-4	100% (826/826 bases)	AY598624	*P. torulosum*	2.4c
5	D2–1	100% (822/822 bases)	AY598626	*P. inflatum*	2.4c
6	A2–1	100% (823/823 bases)	AY598626	*P. inflatum*	1.2c
7	2–3	100% (876/876 bases)	KU210728	*P. ultimum* var. *ultimum*	79.8a
8	?	100% (662/662 bases) 100% (659/659 bases)	KU211001 KT429653	*P. ultimum* var. *ultimum* or *P. ultimum* var. *sporangiferum*	58.3b
9	??	100% (886/886 bases)	KU211001	*P. ultimum* var. *ultimum* or *P. ultimum* var. *sporangiferum*	51.2b
10	IA-Gr-21[z]			*P. irregulare*	14.3c

[x]Results are average of 12 replications with each replication planted with 7 seeds. [y]Means within column followed by the same letter(s) are not significantly different from each other at 5% level of significance ($P < 0.05$). [z]Iowa isolate from Dr. Alison Robertson, Iowa State University

According to Munkvold (2009), seed treatments with strobilurin fungicides have effects on plant physiology. Such fungicides in foliar formulations have played a major role in marketing in some crops, and this has impacted seed treatments as well. Also, some of the trademarks like Plant Health™ (BASF) and Plant Performance™ (Syngenta) have been coined to describe the direct benefits on plant physiology (Munkvold 2009). Similar to strobilurin group, triazoles and other groups of fungicides have an effect on physiology of plants (Tripathi et al. 1980; Wu and Von Tiedemann, 2001). Some of the physiological changes that occur in plants are increased levels of abscisic acid (Grossmann et al. 1999) and antioxidative potential (Wu and Von Tiedemann, 2001), tolerance to environmental stresses (Gerhard et al. 1999; Beck et al. 2002; Reade et al. 2003),

Fig. 4 Pathogenicity test results of *Pythium* spp. in greenhouse. Soybean seeds (cultivar: Pioneer 22T61RR) were planted in white foam cup (237 mL) containing potting mixture mixed with individual *Pythium* isolates fermented on sterilized millet grains. Each isolate was tested in 12 cups and each cup with seven seeds

Table 3 Effects of using commercial foliar fungicides as seed treatments on stand count, vigor, damping-off incidence (%), and yields in a soybean field inoculated with *Pythium ultimum* var. *ultimum* at West Curtiss Research Farm, Iowa State University, Ames, Iowa[x]

Fungicides	Stand count	Plant vigor[y]	Damping-off Incidence (%)	Yield (mt/ha)	Advantage over control	
					Yield (mt/ha)	Economic (USD/ha)[z]
Aproach	551.5a	8.8ba	1.07ba	5.36a	0.31	123.9
Endura	588.0a	9.0a	0.59edc	5.22ba	0.17	67.8
Evito	563.0a	8.8ba	0.62ede	5.20ba	0.15	60.5
Headline	588.3a	9.0a	0.51ed	5.24ba	0.19	74.9
Priaxor	577.5a	9.0a	0.31e	5.33a	0.28	109.4
Quadris	570.0a	8.5ba	0.80bdc	5.31a	0.25	100.9
Stratego YLD	575.3a	8.8ba	0.88bac	5.31a	0.26	103.9
Sercadis	574.5a	8.8ba	0.63edc	5.26ba	0.21	84.2
Control	570.8a	8.3b	1.21a	5.05b	0	0

[x]Results are average of 4 replications each with 16.2 m^2 plot in RCBD design. Means within column followed by the same letter(s) are not significantly different from each other at 5% level of significance ($P < 0.05$). [y]based on a 1–9 scale at growth stage V5, where 1 = poor and 9 = excellent. [z]based on soybean price $397/mt on Sept 30, 2017. (https://ycharts.com/indicators/us_soybean_price_world_bank)

and plant-defense responses (Herms et al. 2002; Conrath et al. 2004).

Yields in plots treated with Endura, Evito, Headline and Sercadis were not significantly different over the untreated control, but certainly these treatments had direct effects in significantly reducing damping off incidence as well as on plant physiology which may have enhanced yield from 0.15 mt/ha in Evito to 0.21 mt/ha in Sercadis treated plots compared with the untreated control (Table 3). On the contrary, plots treated with Aproach and Stratego YLD showed no significant impact on damping off incidence, but certainly showed yield advantage of 0.31 mt/ha and 0.26 mt/ha respectively over the untreated control (Table 3). Significantly, the lowest damping off incidence was observed in Priaxor treated plots but yields were 0.03 mt/ha less than the Aproach. Percent incidence of Pythium damping-off in Aproach treated plots was highest among the fungicides tested plots (0.14% less than the untreated plots), but the yield advantage was also highest among the fungicides tested (Table 3), suggesting that Aproach fungicide may not have direct effect on *Pythium* but of plant performance (Table 3).

There are reports of *Pythium* developing resistance or showing insensitivity to products with an active ingredient of metalaxyl in seed treatments (Dorrance 2014; Anon 2015). Therefore, fungicides tested for soybean seed treatments were labeled for foliar application and were not labeled to control Pythium damping-off. Aproach has not been labeled either foliar or seed treatments against Pythium damping-off, Rhizoctonia root rot and sudden death syndrome. In this study, the product has reduced the incidence of Pythium damping-off by 0.14% and showed 0.31 mt/ha yield advantage compared with the untreated control (Table 3). According to Arysta LifeSciences, Evito foliar spray provides outstanding control of Rhizoctonia aerial blight in addition to several foliar diseases in soybean. In the current study, Evito seed treatments significantly ($P < 0.05$) reduced Pythium damping-off and showed 0.15 mt/ha yield advantage over the untreated control (Table 3).

Of the three BASF products (Headline, Priaxor, and Sercadis) tested, Headline seed treatments significantly ($P < 0.05$) reduced damping-off and showed 0.19 mt/ha yield advantage over the untreated control (Table 3), although it was not labeled for *Pythium* control. According to BASF, Headline, applied in-furrow on corn and soybean, can control soil-borne *Rhizoctonia* while it improves seedling health with more rapid and uniform emergence even under cold and wet conditions. In addition, the EC formulation can be tank-mixed with a liquid fertilizer for easy application. Interestingly, yield advantages in Headline seed treatment are similar to Headline foliar spray either solo or in combination with other fungicides and insecticides across 11 seasons (Navi et al. 2015). However, yield advantages in Headline spray were higher than seed treatments with an average yield advantage of 8.27 mt/ha (range from 3.37 to 13.24 mt/ha), even under low disease pressure (Navi et al. 2015). Although, no significant advantage of Headline (solo or combination) in plots against sudden death syndrome and white mold, significant ($P < 0.05$) yield increase was observed over unsprayed controls indicating plant health benefits of spray (Navi et al. 2015).

Similarly, Priaxor, as a foliar spray is effective against *Alternaria* leaf spot, anthracnose, rust, brown spot, Cercospora blight, frogeye leaf spot, pod and stem blight, Rhizoctonia aerial blight, white mold and southern blight as per the BASF product label. When the product was tested as seed treatment against Pythium damping-off, it significantly reduced the incidence and increased yield by 0.28 mt/ha compared with the untreated control (Table 3). Present yield advantage in Priaxor seed treatment is in agreement with its foliar spray studies in significantly reducing white mold and increasing yields (Navi 2014a, 2014b). Although Sercadis of BASF, is not labeled for soybean diseases but for sheath blight of rice caused by *Rhizoctonia solani*, in seed treatment tests, Sercadis has significantly reduced damping-off incidence with a yield advantage of 0.21 mt/ha (Table 3).

According to Syngenta, Quadris is a broad spectrum, preventative fungicide with systemic and curative properties recommended for the control of many plant diseases. The fungicide can also be applied as a foliar spray in alternating spray programs or in tank mixes with other registered crop protection products. Quadris seed treatment showed significant suppression of damping-off with a yield advantage of 0.25 mt/ha compared with the untreated control (Table 3).

As per Bayer CropScience, Stratego YLD controls Alternaria leaf spot, anthracnose, rust, brown spot, Cercospora blight, frogeye leaf spot, pod and stem blight, powdery mildew, and Rhizoctonia aerial blight. Although foliar spray of Stratego YLD did not show significant effect on sudden death syndrome, white mold and yields (Navi 2013, 2014a), seed treatments in the current study showed significant yield advantage, and 0.33% higher suppression of Pythium damping-off compared with the untreated control (Table 3). Our finding is the first report on the new use of this fungicide. Studies to investigate and strengthen the effect of biocontrol agents on some major yield reducing soil borne pathogens are underway.

Conclusions

In summary, in addition to finding a diversity of *Pythium* species at a single location, we report at least two new *Pythium* species in Iowa. Further studies to identify diversity of species of *Pythium*, *Rhizoctonia*, *Phytophthora* and *Fusarium*, in soils are needed. Most of the foliar fungicides tested for seed treatments show encouraging results. Our findings demonstrate the potential of using foliar fungicides as seed treatments to control Pythium damping-off, which strengthens management options to minimize losses due to seedling diseases with existing products on the market.

Methods

Isolation and identification of *Pythium* spp. from soybean seedlings

Sixty symptomatic soybean seedlings were collected in June 2016 from a planting date × variety maturity study trial at Northeast Research Demonstration Farm, Nashua, Iowa. For surface disinfection, seedlings were rinsed with deionized water, soaked in 20 mL of 1% sodium hypochlorite solution for three minutes in two 9 cm disposable Petri dishes, and rinsed four times in four separate disposable Petri dishes containing sterile deionized water. After disinfection, the seedlings were cut into approximately 1-cm-long pieces using sterilized single edge industrial blade, and transferred with sterile forceps onto Petri dishes of potato dextrose agar (PDA) or PARP (pentachloronitrobenzene 50 mg, ampicillin 250 mg, rifampicin 10 mg and pimaricin 5 mg/L) (Jeffers and Martin 1986), sealed with Parafilm, and incubated at 23 ± 1 °C under a 12 h light/12 h dark cycle for 3–4 days. A total of 30 seedlings were plated on PDA and another 30 on PARP. Filamentous fungal colonies emanating from the peripheral edge of seedling pieces were subcultured on PDA. Each isolate was subcultured and purified by successive hyphal tip transfer. Isolates from PDA and PARP plates were labeled numerically and alphabetically, respectively. Isolates were preliminary characterized based on colony morphology, growth rate (mm/day), and the microscopic observation of oospores and sporangia that are typical of *Pythium* spp.

Putative *Pythium* isolates were grown on PDA for 2–4 days (depending on growth rate) at 23 ± 1 °C, and total DNA was extracted with PrepMan® Ultra and the manufacturer's suggested protocol (Applied Biosystems, Foster City, CA). The nuclear rDNA ITS1–5.8S-ITS2 region (ITS barcode) was amplified using the universal primers ITS6 and ITS4 (Cooke et al. 2000). Sanger Sequencing of amplified DNA was performed by using the same primers at the Iowa State University DNA Facility. Forward and reverse sequences were assembled with Sequence Navigator v 1.0.1 (Applied Biosystems) and compared against the NCBI GenBank using NCBI BLASTn (National Center for Biotechnology Information).

Colony morphological observation and growth rate of *Pythium* isolates

A 6 mm culture plug was taken from the edge of an actively growing colony of a *Pythium* isolate and inoculated onto the center of a PDA plate (9 cm in diameter). Each plate was sealed with parafilm and incubated under a 12 h light/12 h dark cycle at 23 ± 1 °C for 5 days. During the incubation period, mycelial radial growth rate (mm/day) was recorded at 1, 2, 3, 4, and 5 days after plating (DAP). There were 9 treatments, and each treatment had four replications. Variability in colony morphology among the isolates was recorded based on visual and microscopic assessment for the confirmation of the genus *Pythium.*

Pathogenicity tests of *Pythium* spp.

Inoculum from each *Pythium* isolate was reproduced on millet grains by transferring 4 culture plugs to a 500 mL conical flask containing 150 cc of steam-sterilized millet grains and incubated for 10–12 days. Pathogenicity assays of *Pythium* isolates were performed using a previously reported method (Navi and Yang 2016). The pathogenicity of all nine isolates, along with a positive control (*P. irregulare* isolate IA-Gr-21), was tested on soybean variety Pioneer 22T61R in greenhouse conditions in February–March 2017. Twelve cups per isolate were incubated on greenhouse benches in a 16 h/day light under a 400 W E-Ballast, Metal Halide type M59 bulb. During incubation, plants were watered twice daily to maintain moisture in cups. Stand count and damping-off count were recorded at 10 and 20 days after planting. Seedlings with damping-off symptoms were collected and rinsed in running deionized water. Attempts to re-isolate the inoculated *Pythium* spp. were re-performed as described above.

Fungicides evaluation

Stock solution preparation

A total of eight commercial foliar fungicides (Table 4) were evaluated for their effectiveness against Pythium damping-off. Under aseptic conditions, in a pre-disinfected NuAire class II type B2 biological safety cabinet, aliquots of each product (Table 4) were transferred separately to conical flasks containing 1 L sterilized deionized water (SDW). Each of these dilutions was thoroughly mixed by stirring on a Thermolyne magnetic stir plate for two minutes.

Seed treatments

Commercially untreated soybean seed (Pioneer 22T69R) was procured from DuPont Pioneer (1116 Giddings St, Kelley, IA 50134). Five mL of each fungicide stock solution (Table 4) was separately transferred with a sterile syringe to a 33 cm × 39.6 cm Ziploc bag containing 1 kg seed and well-mixed for proper coating of the seeds. Water-treated seed served as a control. The treated and untreated seeds were subsampled at 700 seeds in 8.5 cm × 20.3 cm envelopes per replication of 4-row plots.

Inoculum fermentation

Pythium inoculum was increased on proso millet grains using the fermentation method of Navi and Yang (2016), but with *Pythium* isolates obtained in this study. Fermented *Pythium* inoculum was subsampled at 350 cc per envelope (8.5 cm × 20.3 cm) per 4-row plot (at 5 cc/linear foot).

Planting

To assess efficacy of fungicide seed treatments against damping off, a trial was set up at West Curtiss Research Farm in Ames in a Randomized Complete Block Design (RCBD) with four replications, each 3.0 m × 5.3 m in 76-cm row spacing. Seeds from each individual treatment and the *Pythium* inoculum subsampled separately in envelopes were placed into a 4-row cone plot planter and planted using an ALMACO 4-Row SeedPro Precision Vacuum planter with an automatic cable winding trip system. Post-emergence herbicide was applied to plots when required.

Evaluations

Plots were evaluated for stand counts and plants showing damping-off symptoms at the 2nd and 3rd week after planting (WAP). Also, the plots were evaluated for vigor rating at the V5 growth stage (Fehr et al., 1971; Pederson 2007) on a 1 (poor) to 9 (excellent) vigor rating scale. Disease incidence was calculated by the formula (infected plants × 100) ÷ total plants in each plot.

Table 4 List of foliar fungicides used in seed treatment tests in field against damping-off caused by *Pythium ultimum* var. *ultimum* in a soybean field artificially inoculated

Fungicides	Dilutions used per liter[a]	Labeled spray rates (mL/ha)[b]	Active ingredient (%)	Group name	FRAC code[c]	Manufacturer
Aproach 250SC	3.1 mL	438.2	Picoxystrobin (22.5)	QoI	11	DuPont
Endura*	4.0 g	293.6	Boscalid (70)	SDHI	7	BASF
Evito 480SC	1.0 mL	146.0	Fluoxastrobin (40.3)	QoI	11	Arysta LifeSciences
Headline 250EC	3.1 mL	438.2	Pyraclostrobin (23.6)	QoI	11	BASF
Priaxor 500SC	2.1 mL	292.2	Fluxapyroxad (14.33) + Pyraclostrobin (28.58)	Carboxamides + QoI	7, 11	BASF
Quadris 250FL	3.1 mL	438.2	Azoxystrobin (22.9)	QoI	11	Syngenta
Sercadis 300SC	1.4 mL	197.1	Fluxapyroxad (26.55)	Carboxamides	7	BASF
Stratego YLD	2.1 mL	292.2	Prothioconazole (10.8) + Trifloxystrobin (32.3)	DMI +QoI	3, 11	Bayer

[a]Dilutions were based on the [b]labeled spray rates mixed in 140 L of water. SC = Suspension concentrate, * = Granular; EC = Emulsifiable concentrate, FL = Flowable, QoI fungicides = Quinone outside inhibitors, DMI fungicides = Demethylation inhibitors, SDHI = Succinate dehydrogenase inhibitors, [c]Fungicide Resistance Action Committee

Harvesting

Soybean in different plots was harvested using an ALMACO Research Plot Combine. Yields were adjusted to 13% grain moisture and measured in bushel per acre following the formula, Yield (bushel/acre) = +(100-grain moisture content at harvest) ÷ (100–13) × Harvested grain weight (pound) per plot ÷ 60 ÷ (plot length ft. × plot width ft.) ÷ 43,560. Where, 100–13 is adjusted grain moisture content to 13%, 60 = pounds per bushel, 43,560 = area per acre in ft^2.

Subsequently, the yields in bushel/acre from the above were converted to mt/ha following the formula; Yield (mt/ha) = Yield (bushel/acre) × 0.0272155 × 2.47. Where, 0.0272155 is the weight in metric ton per bushel (US grains council https://grains.org/markets-tools-data/tools/converting-grain-units/), and 2.47 is conversion from 1 ha to acres.

Tillage and weed management

At West Curtiss Farm, fields received Generic Prowl at 2.9 l/ha and 0.3 l/ha of Sonic on May 6th and field cultivated the same day. Post-planting on June 22, sprayed 1.0 l/ha Clethodim and 1.2 l/ha of Flexstar. Fields at West Curtiss got a 21–70–120-17.5-1.75 (N-P-K-S-Zn) in fall.

Data analysis

Effects of fungicide seed treatments on soybean stand counts, plant vigor, incidence of Pythium damping-off, and grain yields were analyzed using PROC ANOVA in SAS 9.4. (SAS, LLC, Cray, NY). Fisher's least significant difference was used to detect the significant differences among the means ($P < 0.05$).

Abbreviations

ANOVA: Analysis of variance; DAP: Days after planting; DMI: Demethylation inhibitors; NCBI: National Center for Biotechnology Information; PARP: Pimaricin, ampicillin, rifampicin, pentachloronitrobenzene; PDA: Potato dextrose agar; QoI: Quinone outside inhibitors; RCBD: Randomized complete block design; SDHI: Succinate dehydrogenase inhibitors; SDW: Sterilized deionized water; WAP: Week after planting

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The first two authors processed diseased samples for pathogen isolation, conducted greenhouse and field tests, the third author did sequencing and the fourth author supervised the project. It was a team work and all authors shared their respective expertise in drafting and approved the final draft of the manuscript for submission.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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