Pathogenicity and Virulence of Soilborne Oomycetes on *Phaseolus vulgaris*

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Abstract

Dry bean (*Phaseolus vulgaris* L.) is a globally important leguminous food crop. Yields can be reduced by high incidence of soilborne oomycetes that cause seedling disease. Breeders have attempted to develop *Pythium* root rot-resistant bean varieties; however, relationships between dry bean and most soilborne oomycete species remain uncharacterized. Oomycete species ($n = 28$), including *Pythium* spp. and *Phytophthora* spp., were tested in a growth chamber seedling assay at 20°C and an in vitro seed assay at 20°C and 26°C to evaluate their pathogenicity and virulence on 'Red Hawk' dark red kidney bean and 'Zorro' black bean. Root size or disease severity was significantly impacted by 14 oomycete species, though results varied by bean variety, temperature, and assay. Of these 14 pathogenic

oomycete species, 11 species exhibited significant differences in DSI due to temperature on at least one bean variety. *Pythium aphanidermatum*, *P. myriotylum*, *P. ultimum*, *P. ultimum* var. *sporangiferum*, and *P. ultimum* var. *ultimum* were the most virulent species in both assays, causing seed rot and pre-emergence damping-off of dry bean. Oomycete species were clustered into three groups based on symptom development: seed rot pathogens, root rot pathogens, or nonpathogens. Intraspecific variability in virulence was observed for eight of the 14 pathogenic oomycete species. Improved understanding of *Pythium* and *Phytophthora* interactions with dry bean may enable breeders and pathologists to more effectively evaluate strategies for oomycete seedling disease management.

The most important food legume is dry bean (*Phaseolus vulgaris* L.), accounting for more than half of all food legume production internationally (Miklas et al. 2006). Dry bean was domesticated in Central America and the Andes mountains. Dry bean varieties originating from these regions have been classified into two respective gene pools, the Middle American and the Andean (Bitocchi et al. 2013; Gepts 1988). Although the majority of global dry bean production occurs in Latin America and East Africa (Blair 2013; Broughton et al. 2003; Miklas et al. 2006), dry bean is planted on substantial land area in Michigan and other parts of temperate North America (National Agricultural Statistics Service 2015). Bean yields may be compromised by seedling disease and root rot as a result of *Pythium* and *Phytophthora* species (Blair et al. 2010; Broughton et al. 2003; Hoch and Hagedorn 1974; Navarro et al. 2008).

Pythium and *Phytophthora* (formerly *Pythium* Clade K) (de Cock et al. 2015) are ubiquitous genera in the oomycetes that together may contain as many as 300 species (Schroeder et al. 2013), many of which are plant pathogens. *Pythium* is divided into 10 clades based on genetic and morphological differences (Robideau et al. 2011). Oomycete species from these genera may reduce yield of dry bean (Li et al. 2014; McCarter and Littrell 1970; Nzungize et al. 2011) and other field crops (Broders et al. 2007; Higginbotham et al. 2004; Rojas et al. 2017) by causing pre- and postemergence damping-off of seedlings or root rot (Hendrix and Campbell 1973). Severity of disease may vary among oomycete isolates and species, environments, and hosts (Broders et al. 2009; Matthiesen et al. 2016; Olson et al. 2016; Rojas et al. 2017; Roncadori and McCarter 1972; Wong et al. 1984). Though 30 *Pythium* and 4 *Phytophthora* species have been associated with dry bean (USDA-ARS 2016), the pathogenicity and virulence of

these and other oomycete species remain largely uncharacterized. Studies in Australia and East Africa have evaluated the pathogenicity of multiple regionally important species (Gichuru et al. 2016; Li et al. 2014; Nzungize et al. 2011), but a study evaluating pathogenicity of many regionally important *Pythium* and *Phytophthora* species on dry bean has not been conducted in North America.

Root rot-resistant bean varieties and seed-applied fungicides are commonly utilized in dry bean production for management of pathogenic oomycete species (Abawi and Corrales 1990; Keinath et al. 2000). Fungicide seed treatments have been successful in reducing stand and yield losses in dry bean (Keinath et al. 2000; Locke et al. 1983). However, organic certification regulations (Koch et al. 2010) and observed oomycete fungicide insensitivity (Broders et al. 2007; Falloon et al. 2000; Matthiesen et al. 2016; Mazzola et al. 2002; Munera and Hausbeck 2016; Taylor et al. 2002) indicate that breeding for resistance to *Pythium* root rot remains vital for improving disease management in dry bean. Dry bean varieties from the Andean gene pool have been considered to be more susceptible to fungal root rot pathogens, such as *Fusarium* spp., than Middle American varieties (Blair et al. 2010; Conner et al. 2014), likely because certain genes conferring root rot resistance to fungi occur with more frequency in Middle American varieties than in Andean varieties (Nicoli et al. 2011; Román-Avilés and Kelly 2005; Schneider et al. 2001). However, resistance to *Fusarium* root rot is inherited independently from resistance to *Pythium* root rot in dry bean (Ongom et al. 2012). Consequently, it remains unclear how the genetic differences between dry bean gene pools influence resistance to oomycete diseases such as *Pythium* root rot, especially since resistance of dry bean to a single pathogenic oomycete species may not confer resistance to all other oomycete species (Binagwa et al. 2016). Consequently, evaluation of resistance in dry bean varieties may be best determined by testing across pathogenic oomycete species.

Since temperatures and edaphic conditions have been shown to impact oomycete growth and virulence (Broders et al. 2007, 2009; Cantrell and Dowler 1971; Pieczarka and Abawi 1978; Rojas et al. 2017; Wei et al. 2010), more descriptive evaluations of pathogenicity and virulence may be attained by using multiple temperatures and assays. By determining oomycete species that are most problematic for dry bean production, bean breeders will be equipped to identify and develop bean varieties with resistance to the most virulent species. Additionally, pathologists may be able to better evaluate seed treatments and other management practices for *Pythium* root rot, leading to improved disease management recommendations in dry bean production.

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To better understand the major oomycete diseases of dry bean, a study was conducted to i) determine the pathogenicity and comparative virulence of select oomycetes from the Midwestern United States and Uganda on dry bean, ii) compare the severity of oomycete disease on dry bean varieties from the Middle American and Andean gene pools, and iii) examine the virulence of oomycete species on dry bean seed at two temperatures.

Materials and Methods

Two dry bean varieties commonly planted in Michigan were selected to evaluate severity of *Pythium* and *Phytophthora* seed and root rot: ‘Red Hawk’ kidney bean (Kelly et al. 1998) of the Andean gene pool and ‘Zorro’ black bean (Kelly et al. 2009) of the Middle American gene pool. Dry bean seeds were obtained from the Michigan State University Dry Bean Breeding Program.

A total of 85 isolates representing 28 oomycete species were tested for pathogenicity on dry bean. These 28 oomycete species included two *Phytophthora* species and 26 *Pythium* species. Three arbitrarily selected isolates of each species were evaluated in the study unless otherwise noted (Table 1). These oomycete isolates were identified by sequencing the ITS region using ITS4 and ITS6 primers (Cooke et al. 2000; White et al. 1990) and blasting against a curated database (Robideau et al. 2011; Rojas et al. 2017; Jacobs and Chilvers, unpublished).

In order to evaluate oomycete species that may be most problematic for dry bean production, *Pythium* and *Phytophthora* species were selected based on abundance or disease severity data from previous

oomycete community surveys conducted in our lab (Rojas et al. 2017; Jacobs and Chilvers, unpublished). Oomycete species included in this study were recovered in the surveys at a rate above 2.5% or were known pathogens of dry bean (USDA-ARS 2016). Isolates were collected from soybean seedlings across the Midwestern United States ($n = 57$), dry bean seedlings in Michigan ($n = 24$), and from dry bean seedlings in Uganda ($n = 4$) (Table 1).

Pathogenicity assays. A growth chamber seedling assay and an in vitro seed assay were conducted to evaluate the pathogenicity and virulence of oomycete species on dry bean. For both assays, the 85 isolates were arbitrarily divided into seven groups, denoted as “experiments.” Each experiment included 12 *Pythium* or *Phytophthora* isolate treatments, respective noninoculated controls (NIC), and an inoculated control (IC) treatment using an isolate of *Pythium ultimum* var. *ultimum* (MISO_8-10) known to be highly virulent. The seedling assay had two NIC treatments, one with noninoculated rice and the other without rice. Each experiment consisted of three repeated trials, and each trial included three treatment replications. Thus, each isolate treatment was replicated nine times across experiments.

Seedling assay. Oomycete-colonized rice was used as inoculum. Inoculum was prepared by mixing 20 ml of dH₂O with 30 g of par-boiled white rice in a 125-ml Erlenmeyer flask. Flasks were covered with aluminum foil and autoclaved for 30 min. To prevent cohesion of rice grains after autoclaving, rice grain aggregates were aseptically broken apart by carefully percussing flasks against rolls of paper towel, and flasks were autoclaved again for 30 min after cooling to room temperature, typically overnight. Isolates of *Pythium* species were grown on semiselective corn meal agar (CMA-PARPB) amended to have a final concentration of 10 µg/ml pimaricin, 250 µg/ml ampicillin, 10 µg/ml rifampicin, 50 µg/ml pentachloronitrobenzene, and 10 µg/ml benomyl (Jeffers and Martin 1986; Rojas et al. 2017). Five 0.6-cm diameter plugs were cut from the leading edge of 2- to 3-day-old mycelial growth using a flame-sterilized cork borer and transferred to each rice-filled flask. Inoculated rice was incubated at room temperature for 10 to 14 days depending on rate of mycelial growth, and rice grain aggregates were broken apart every 2 to 3 days as previously described.

Four 4-mm diameter drainage holes were made in the bottom of 354-ml insulated paper cups (Solo Cup Company, Lake Forest, IL). Cups were filled with the following layers: 200 ml of medium vermiculite, 7 g of inoculum (except for the noninoculated control), 70 ml of medium vermiculite, six dry bean seeds, and 70 ml of medium vermiculite. Cups were placed into a growth chamber and watered with half-strength Hoagland solution (Hoagland and Arnon 1938) until water dripped from the bottom. Dry beans were grown at 20°C and 85% relative humidity with 14 h of light at illuminance of 500 to 590 µE m⁻² s⁻¹ and 10 h of darkness each day. After 12 days, dry bean emergence was recorded. Roots were then washed with tap water and scanned at 300 dpi resolution on a flatbed scanner. Total root area and root length of each plant was determined from the scanned root images using Assess 2.0 software (American Phytopathological Society, St. Paul, MN). After being scanned, roots were separated from shoots and dried in a forced-air drying oven at 38°C until dry weight stabilized. Root dry weight was measured and recorded.

Koch’s postulates were fulfilled for the seedling assay by reisolating the oomycete species from dry bean roots. Within both dry bean varieties, a representative plant was selected from each isolate treatment and approximately 1 cm of darkened root tissue was plated onto CMA-PARPB to reisolate the original isolate used for inoculation. Reisolations were performed once per trial for a total of three times for each isolate treatment. The original cultures used for inoculation and the cultures reisolated from root samples were transferred to CMA-PARPB and grown out for 2 to 3 days. A crude DNA extraction was performed by collecting pinhead sized mycelia pieces with a sterile wooden toothpick, placing mycelia into a 1.5-ml centrifuge tube containing 100 µl of sterile filtered water, and incubating the tube contents at 94°C in a heat block for 10 min. Samples were cooled on ice for at least 5 min and were temporarily transferred to a -20°C freezer or used immediately for DNA amplification. PCR amplification was conducted in 25-µl reactions containing 19.1 µl

Table 1. Information of isolates used to determine pathogenicity and virulence of soilborne oomycete species on dry bean

Species	N	Clade ^a	Host ^b	Location ^c
<i>P. aphanidermatum</i>	3	A	DB, SB	IL, NE, MI
<i>P. deliense</i>	1	A	DB	U
<i>P. aff. diclinum</i>	3	B	SB	IA, ND, ND
<i>P. aff. dissotocum</i>	3	B	SB	AR, MI, IA
<i>P. aff. torulosum</i>	3	B	DB, SB	MI, MI, IA
<i>P. CAL_2011f</i>	3	B	DB	MI, MI, MI
<i>P. coloratum</i>	3	B	DB, SB	MI, MI, MN
<i>P. conidiophorum</i>	3	B	SB	IA, IL, NE
<i>P. inflatum</i>	3	B	DB	MI, MI, MI
<i>P. lutarium</i>	2	B	SB	IN, MI
<i>P. myriotylum</i>	3	B	DB	MI, MI, MI
<i>P. oopapillum</i>	3	B	SB	IL, IN, MI
<i>P. pachycaule</i>	3	B	SB	MN, NE, IA
<i>P. torulosum</i>	3	B	SB	MN, MI, MI
<i>P. acanthicum</i>	2	D	SB	KS, MI
<i>P. rostratiformis</i>	3	E	SB	IA, MI, NE
<i>P. attrantheridium</i>	3	F	DB, SB	MI, MI, IA
<i>P. irregulare</i>	3	F	DB, SB	MI, KS, WI
<i>P. paroecandrum</i>	3	F	SB	AR, IN, AR
<i>P. spinosum</i>	4	F	SB	AR, AR, IN, IN
<i>P. sylvaticum</i>	8	F	DB, SB	IN, ND, NE, MI, MI, MI, MI, MI
<i>P. heterothallicum</i>	3	I	DB, SB	MI, MI, IA
<i>P. ultimum</i>	5	I	DB, SB	U, U, IL, KS, MN
<i>P. ultimum</i> var. <i>sporangiiferum</i>	3	I	SB	IL, IN, KS
<i>P. ultimum</i> var. <i>ultimum</i>	3	I	SB	MI, IL, WI
<i>P. perplexum</i>	3	J	SB	ND, NE, MI
<i>Phytophthora</i> aff. <i>vexans</i>	2	K	DB, SB	MI, KS
<i>Phytophthora cucurbitacearum</i>	1	K	DB	U

^a Clades were determined from Robideau et al. 2011; isolates marked “K” are the genus *Phytophthora*.

^b Host from which *Pythium* isolates were originally collected include DB: dry bean or SB: soybean.

^c Abbreviations for locations of isolate collection include the following: AR, Arkansas; IA, Iowa; IL, Illinois; IN, Indiana; KS, Kansas; MI, Michigan; MN, Minnesota; ND, North Dakota; NE, Nebraska; U, Uganda; and WI, Wisconsin.

of sterile filtered water, 2.5 µl of 10× DreamTaq buffer, 0.2 µl of 25 mM dNTP, 0.5 µl of 10 µM primers ITS6 and ITS7, 0.2 µl of 5U/µl DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA), and 2 µl of crude DNA. PCR parameters consisted of 94°C for 3 min; 35 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 1 min; 72°C for 7 min; and hold at 4°C. The PCR products were utilized for single-strand confirmation polymorphism (SSCP) as described by Kong et al. (2004, 2005). DNA from original cultures and reisolated cultures were run side by side in an 8% polyacrylamide gel to compare banding patterns. If banding patterns of the reisolated culture matched the banding pattern of the original culture in at least two of three trials and disease symptoms were observed, the oomycete isolate was considered to be pathogenic on dry bean.

Seed assay. The same oomycete isolates and dry bean varieties used in the seedling assay were also utilized in an in vitro seed assay. The seed assay was conducted at 20°C and 26°C, which corresponds to the normal maximum daytime air temperatures in Michigan during May and June, respectively (Prism Climate Group, Oregon State University 2016). From oomycete isolates cultured on CMA-PARPB, a 0.6-cm diameter plug from the leading edge of mycelial growth of each isolate was transferred onto the center of water agar in a 100-mm Petri dish. Cultures were incubated in the dark at one of the two treatment temperatures. Dry bean seeds were surface disinfected by soaking them in a 0.50% sodium hypochlorite solution. Based on preliminary trials (data not shown), dry bean varieties Red Hawk and Zorro were surface disinfected for 20 min and 10 min, respectively, triple-rinsed with sterile deionized water, and air-dried on sterile paper towel in a biosafety cabinet. After 2 days of incubation, cultures on water agar were removed from controlled-temperature conditions, and 10 dry bean seeds were placed in a circle 5 mm away from the edge of each Petri dish. Due to differences in seed size, Petri dishes were 100 mm × 20 mm and 100 mm × 15 mm for Red Hawk and Zorro seeds, respectively. Petri dishes with Red Hawk and Zorro seed were then returned to the respective temperature conditions and incubated for an additional 7 days. Each seed was visually evaluated and rated for seed rot and root rot using a modified disease severity scale (Broders et al. 2007; Rojas et al. 2017): 0 = seed germinated without symptoms, 1 = seed germinated with visibly reduced growth yet without visible lesions, 2 = seed germinated with reduced growth and lesions, 3 = seed germinated with coalesced lesions on root tips or root surface at least partially colonized by visible mycelial growth, and 4 = no germination or complete colonization of seed by oomycete mycelia.

Statistical analysis. Data were analyzed using proc mixed from SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC). Pearson correlations were conducted using the Hmisc package (Harrell 2016) in R version 3.2.3 (R Core Team 2015).

In the seedling assay, the presence or absence of inoculum substrate resulted in no significant differences between the NIC with rice and the NIC without rice. Though all isolates were not evaluated simultaneously, the presence of control treatments in each experiment made it possible to compare across varieties by normalizing the data in reference to the NIC with rice. Disease severity in the seedling assay was determined by normalizing emergence and root size within each trial to be expressed as a percentage of the “NIC with rice” data means. Oomycete species, dry bean variety, and their interaction were treated as fixed effects while random effects included isolate nested within the interaction among oomycete species, trial, and experiment (Table 2).

Cluster analysis was performed for each dry bean variety to group *Pythium* and *Phytophthora* species together based on similarities in symptom severity. The least square means of the root dry weight, root length, root area, and seedling emergence data from the seedling assay were scaled and utilized as proxies for virulence. Cophenetic distances were determined using the cord distance (CRD) method (Foster and Bills 2011), resulting in cophenetic correlations and root mean square errors above 0.95. The CRD method was specified within the pheatmap R package to build a dendrogram and heat map (Kolde 2015).

In the seed assay, disease severity ratings were converted to a continuous disease severity index (DSI) by calculating the sum of disease severity ratings for the 10 seeds and dividing by 40 (the highest

possible rating for 10 seeds), as performed previously (Li et al. 2014; Rojas et al. 2017). Oomycete species, dry bean variety, temperature, and all significant interaction effects were treated as fixed effects while random effects included isolate nested within the interaction among oomycete species, experiment, and trial (Table 3).

Results

Previous host of isolates used in the current study had no significant overall effect on virulence for any parameters measured in the seedling assay ($P > 0.10$). The effect of previous host was significant for DSI in the seed assay ($P = 0.0115$), in which oomycete species isolated from dry bean tended to be slightly more virulent than oomycete species isolated from soybean.

Seedling assay. Oomycete species varied in the severity of disease induced on dry bean. Though many oomycete species caused no symptoms, all oomycete species evaluated were successfully reisolated from inoculated plants in at least two of three instances. Across both dry bean varieties, 11 *Pythium* species significantly reduced the emergence, root dry weight, root area, or root length relative to the NIC with rice in the seedling assay (Fig. 1). Six of these *Pythium* species (*P. aphanidermatum*, *P. myriotylum*, *P. spinosum*, *P. ultimum*, *P. ultimum* var. *sporangiferum*, and *P. ultimum* var. *ultimum*) caused pre-emergent damping-off and significantly reduced emergence and all root parameters by $\geq 25\%$. These six species were determined to be seed rot pathogens. The other five *Pythium* species (*P. aff. diclinum*, *P. CAL_2011f*, *P. irregulare*, *P. paroecandrum*, and *P. sylvaticum*) caused visible root rot symptoms and significant reductions of $\geq 25\%$ relative to the NIC with rice for at least two of the three root parameters: root dry weight, root area, and root length. These five oomycete species were determined to be root rot pathogens, though significant root area reduction varied substantially from 31% (*P. paroecandrum*) to 89% (*P. irregulare*). The remaining 17 oomycete species did not significantly impact seedling assay parameters. These three symptom groups generally corresponded to the cluster analysis results, in which the oomycete species were clustered into three groups relating to symptom type and severity for both Red Hawk (Fig. 2) and Zorro (Fig. 3). Oomycete species that significantly reduced emergence were considered “seed rot” pathogens; species that significantly reduced root area, dry weight, or length but did

Table 2. Analysis of variance for seedling assay parameters

Assay parameters ^a	MSE	F	P-value
Emergence			
Treatment	58,673	48.60	<0.0001
Variety	61	0.32	0.5738
Treatment*variety	1,149	5.97	<0.0001
Isolate (species*experiment*trial)	1,185	6.15	<0.0001
Residual	192		
Root dry weight			
Treatment	75,142	46.08	<0.0001
Variety	17,577	55.64	<0.0001
Treatment*variety	2,002	6.34	<0.0001
Isolate (species*experiment*trial)	1,602	5.07	<0.0001
Residual	316		
Root area			
Treatment	91,619	38.99	<0.0001
Variety	4,079	9.44	0.0022
Treatment*variety	3,226	7.47	<0.0001
Isolate (species*experiment*trial)	2,307	5.34	<0.0001
Residual	432		
Root length			
Treatment	92,407	52.56	<0.0001
Variety	12,186	44.36	<0.0001
Treatment*variety	1,429	5.20	<0.0001
Isolate (species*experiment*trial)	1,725	6.28	<0.0001
Residual	275		

^a Isolate nested within the interaction between species, experiment, and trial was the only random effect.

not reduce emergence were considered “root rot” pathogens; and all other species were considered nonpathogens. *P. aff. diclinum*, *P. irregulare*, *P. paroecandrum*, and *Phytophthora* aff. *vexans* clustered into different groups depending on the dry bean variety.

Virulence of oomycete isolates was found to vary significantly within eight species. For example, variability among *P. sylvaticum* isolates ($n = 8$) and *P. spinosum* isolates ($n = 4$) was highly significant ($P < 0.0001$) for root area, root length, and root dry weight in the seedling assay. Six additional oomycete species, each represented by three isolates, also had significant differences among isolates for root area, root length, and root dry weight (Table 4). Within *P. spinosum*, the two isolates with significantly greater virulence were isolated from the state of Arkansas, whereas the other two *P. spinosum* isolates were collected in the state of Indiana.

Though the main effect of dry bean variety on emergence was non-significant ($P = 0.5737$), the effect of oomycete species on emergence varied significantly different between the two bean varieties as indicated by the significant interaction between dry bean variety and oomycete treatment ($P < 0.0001$) (Table 2). Similarly, when

oomycete species were categorized as causing seed rot, root rot, or no disease, the two dry bean varieties responded dissimilarly to each group. For example, emergence of Zorro was significantly higher than Red Hawk when challenged with the inoculated control ($P < 0.0001$), but emergence of Red Hawk was significantly higher than Zorro when challenged with root rot pathogens ($P = 0.0218$) (Supplementary Table S1).

Expressed as percentages relative to the NIC, the root dry weight, area, and length of Zorro was significantly higher than Red Hawk across all treatments ($P < 0.0001$, $P = 0.0022$, and $P < 0.0001$, respectively) (Table 2). However, the dry bean varieties had significantly different root parameter responses to some oomycete species as indicated by the significant interaction between dry bean variety and oomycete treatment ($P < 0.0001$ for all root parameters) (Table 2). For example, *P. paroecandrum* caused significant reductions in root dry weight and root area for Red Hawk, but not for Zorro. In the seedling assay, at least two root parameters were significantly reduced by 11 and 10 oomycete species on Red Hawk and Zorro, respectively (Supplementary Table S2).

Oomycete species were grouped by symptom type (seed rot, root rot, or nonpathogen) and by clade, and the responses of the dry bean varieties to these groups were found to differ significantly from one another. Zorro root dry weight and root length was significantly greater than Red Hawk for nonpathogenic oomycete species ($P < 0.0001$ for both). Though Zorro had significantly higher root area than Red Hawk when challenged with seed rot pathogens ($P = 0.0395$), Red Hawk challenged with root rot pathogens had significantly higher emergence than Zorro ($P = 0.0093$). Similarly, when *Pythium* species were divided by clade (*Phytophthora* being treated as its own “clade”), bean varieties were found to have differences in susceptibility to oomycetes from particular clades (Supplementary Table S3). Relative to the respective NIC, the root area, length, and dry weight of Red Hawk was significantly lower than Zorro for *Pythium* Clade A ($n = 4$ isolates) and *Phytophthora* ($n = 3$ isolates). When challenged with *Pythium* Clade F ($n = 21$ isolates), Red Hawk had significantly greater emergence than Zorro ($P < 0.0001$), but Zorro had significantly greater root area ($P = 0.0086$).

Table 3. Analysis of variance for seed assay parameters

Assay parameters ^a	MSE	F	P-value
DSI			
Treatment	0.30	22.53	<0.0001
Variety	0.02	2.23	0.1352
Treatment*variety	0.03	3.16	<0.0001
Temperature	0.05	4.48	0.0345
Treatment*temperature	0.10	9.54	<0.0001
Variety*temperature	0.01	0.73	0.3943
Treatment*variety*temperature	0.03	3.07	<0.0001
Isolate (species*experiment*trial)	0.19	17.87	<0.0001
Residual	0.01		

^a Isolate nested within the interaction between species, experiment, and trial was the only random effect.

Fig. 1. Emergence, root dry weight, root length, and root area of dry bean inoculated with oomycete species in the seedling assay at 20°C, across two dry bean varieties, expressed as a percentage relative to the noninoculated control (NIC) with rice. The NIC is marked by the gray arrow. The inoculated control was an isolate of *Pythium ultimum* var. *ultimum* known to be virulent on bean. Gray bars indicate species that caused significant reductions in emergence or root size relative to the NIC by Tukey’s HSD, $\alpha = 0.05$. White bars indicate no significant difference.

Seed assay. Nine oomycete species were found to elicit a significantly greater DSI than the NIC across dry bean varieties and temperatures (Fig. 4). *Pythium aphanidermatum*, *P. myriotylum*, *P. ultimum*, *P. ultimum* var. *sporangiferum*, and *P. ultimum* var. *ultimum* caused DSI above 0.60, which indicated visible seed rot symptoms. *P. CAL_2011f*, *P. deliense*, *P. irregulare*, *P. spinosum*, and *P. sylvaticum* had DSI between 0.3 and 0.6, indicating the presence of visible root rot lesions on germinated seeds. The remaining 18 oomycete species had DSI values below 0.3, indicating that germinated seeds lacked consistently visible symptoms. DSI caused by the oomycete species varied significantly by dry bean variety and by temperature (Table 3).

In the seed assay, virulence of oomycete isolates was found to vary significantly within species (Table 3). The two *P. ultimum* isolates from Uganda caused significantly higher DSI than the three isolates from the Midwest ($P < 0.0001$). Similarly, variability among *P. spinosum* isolates ($n = 4$) was highly significant ($P < 0.0001$) for DSI in the seed assay, and five additional oomycete species, *P. aphanidermatum* ($n = 3$), *P. CAL_2011f* ($n = 3$), *P. conidiophorum* ($n = 3$), *P. paroecandrum* ($n = 3$), and *P. sylvaticum* ($n = 8$), had significant intraspecific variation in seed assay DSI as well as in seedling assay root parameters (Table 4). Behavior of oomycete isolates was quite consistent across both assays, with high correlations between DSI from the seed assay and each of the parameters measured from the seedling assay (for all correlations, $P < 0.0001$ and Pearson's $r > 0.85$) (Supplementary Fig. S1).

Across seed assay treatments, DSI measured on Zorro was significantly greater than DSI on Red Hawk ($P < 0.0001$). When evaluated

by clade, *Phytophthora* was the only group of species for which DSI on Red Hawk was numerically, though not significantly, higher than on Zorro ($P = 0.7805$). Temperature also had a significant overall effect on DSI, with higher DSI across dry bean varieties at 26°C than at 20°C ($P = 0.0345$) (Table 3). On Red Hawk, 10 and nine oomycete species caused significantly higher DSI than the NIC at 20°C and 26°C, respectively (Fig. 4). On Zorro, 10 and 12 oomycete species caused significantly higher DSI than the NIC at 20°C and 26°C, respectively. In the seed assay, 14 oomycete species caused significantly different DSI at 20°C than at 26°C on at least one dry bean host (Fig. 4). On both Red Hawk and Zorro, three oomycete species caused significantly higher DSI at 20°C than 26°C, whereas four other oomycete species, including *P. deliense*, caused significantly higher DSI at 26°C. Though *Phytophthora* aff. *vexans* and *Pythium paroecandrum* had significantly higher DSI at 20°C than at 26°C on Red Hawk, DSI for both species were significantly higher at 26°C than at 20°C on Zorro. The DSI of five other oomycete species responded significantly to temperature on only one bean variety. When evaluated across bean varieties, the DSI caused by oomycete isolates within a species tended to respond similarly to temperature, but *P. paroecandrum* and *P. ultimum* each included at least one isolate with significantly greater virulence at 20°C than 26°C ($P < 0.0001$ and $P = 0.0004$, respectively) and at least one isolate with significantly greater virulence at 26°C than 20°C ($P < 0.0001$ for each isolate). Multiple oomycete clades represented in the study contained isolates with similar virulence responses to temperature. *Pythium* Clade A ($n = 4$) and genus *Phytophthora* ($n = 3$) had significantly

Fig. 2. Red Hawk kidney bean cluster analysis for virulence of *Pythium* and *Phytophthora* species using seedling assay data parameters. Blue, yellow, and red represents low, moderate, and high disease pressure, respectively. "NIC, Rice" and "NIC, No Rice" refer to the noninoculated controls with and without rice substrate, respectively.

higher DSI at 26°C than 20°C, whereas *Pythium* Clade F ($n = 21$) exhibited the opposite trend (Supplementary Table S4).

Discussion

Findings from the current study support previous studies demonstrating that many *Pythium* species cause seedling disease in dry bean and that dry bean variety and temperature can influence disease severity (Li et al. 2014; Nzungize et al. 2011; Pfender 1981; Pieczarka and Abawi 1978; Sippell and Hall 1982). For both dry bean varieties, the same nine *Pythium* species caused significant differences from the NIC for root area, root length, root weight, and seed assay DSI. However, the pathogenicity of three additional *Pythium* species depended on temperature, dry bean variety, and assay.

This study constitutes the first report of *P. CAL_2011f* and *P. deliense* as pathogens of dry bean. *P. CAL_2011f* of Clade B was found to cause moderate root rot under all experimental conditions of this study. In the seed assay, *P. CAL_2011f* was significantly more virulent at 20°C than 26°C. *P. deliense* was found to cause disease only in the seed assay. *P. aff. diclinum* was also found to cause disease on dry bean at 20°C in the seedling assay. Though *P. aff. diclinum* has not been explicitly reported to cause disease on dry bean, *P. diclinum sensu stricto* has been (Nzungize et al. 2011). The nine other *Pythium* species that exhibited pathogenicity in this study have been previously reported as pathogens of dry bean, including *P. aphanidermatum*, *P. irregulare*, *P. myriotylum*, *P. paroecandrum*, *P. spinosum*, *P. sylvaticum*, *P. ultimum*, *P. ultimum* var. *sporangiferum*, *P. ultimum* var. *ultimum*, and *Phytopythium* aff.

vexans (Kobriger and Hagedorn 1984; Li et al. 2014; Nzungize et al. 2011; Olson et al. 2016; Papias et al. 2016).

Disease severity of root rot pathogens was highly variable. In the cluster analysis, the highly virulent *P. irregulare*, despite having no impact on emergence, caused severe root rot and was grouped with either the “root rot” or “seed rot” pathogens depending on bean variety. Similarly, pathogens that caused minor root rot, such as *P. aff. diclinum* and *P. paroecandrum*, were sometimes grouped either as a nonpathogen or a root-rot pathogen depending on bean variety. The visual rating scale for DSI used in this study did not fully align with the seedling assay in identifying *P. aff. diclinum* as a pathogen and therefore may not always sufficiently describe the pathogenicity and virulence of less virulent oomycete pathogens. However, the seed assay remains useful for identifying oomycete species that can cause readily visible root rot or seed rot. The sensitivity of the seed assay may have been lower than the seedling assay due to variation in humidity, the difference between using rice inoculum and CMA-PARPB, seedborne pathogens that are not subject to surface sterilization (Marcenaro and Valkonen 2016), and dissimilar rate of seedling development of the two bean varieties.

Previous studies in Rwanda and Australia have found that *Phytophthora cucurbitacearum*, *P. conidiophorum*, *P. lutarium*, *P. pachycaule*, and *P. rostratifingens* were able to cause disease in dry bean (Li et al. 2014; Nzungize et al. 2011). However, isolates from the *Pythium* species used in the current study did not significantly increase DSI or reduce root size or emergence relative to the NIC on either dry bean variety screened. Though these oomycete species

Fig. 3. Zorro black bean cluster analysis for virulence of *Pythium* and *Phytopythium* species using seedling assay data parameters. Blue, yellow, and red coloration represents low, moderate, and high disease pressure, respectively. "NIC, Rice" and "NIC, No Rice" refer to the noninoculated controls with and without rice substrate, respectively.

previously reported as pathogens may have asymptotically colonized plant roots, they did not exhibit pathogenicity in the present study, either because the particular isolates evaluated were not pathogenic or because of differences in experimental conditions such as temperature, humidity, pH, or dry bean cultivar (Martin and Loper 1999; Rojas et al. 2017). Though some oomycete species have exhibited differential zoospore accumulation or virulence across hosts (Augsburger and Wilkinson 2007; Ingram and Cook 1990; Mitchell and Deacon 1986), the original host of the *Pythium* isolates evaluated in this study had no significant effect on emergence, root dry weight, root area, and root length.

Though virulence was not significantly different among isolates for most *Pythium* species, significant intraspecific variability was observed in the current study, as has been reported previously (Broders et al. 2007; Higginbotham et al. 2004; Olson et al. 2016; Zhang and Yang 2000). Intraspecific variability in root area, root length, root weight, and DSI was observed within *P. sylvaticum* ($n = 8$), *P. spinosum*, and four other oomycete species. Selection pressures from different crop rotations (Zhang and Yang 2000), nutritional status of an isolate (Nelson and Hsu 1994), isolate age, or concentration of isolate propagules (Granke and Hausbeck 2010) may contribute to the intraspecific variability observed in the current study. Intraspecific variability may also be related to origin of an oomycete isolate, as both *P. spinosum* isolates collected from Arkansas were highly virulent seed rot pathogens, whereas the other two *P. spinosum* isolates collected from Indiana were much less virulent and seemed to have little effect on dry bean emergence. Though intraspecific differences in virulence may be related to isolate origin, we were unable to adequately evaluate geographic effects on isolates virulence because relatively few isolates were used from each species.

In contrast to the results of some previous studies (Li et al. 2014; Nzungize et al. 2011), different bean varieties responded differently to different pathogens. Relative to their respective NIC, Zorro had higher emergence than Red Hawk when challenged with the inoculated control (*P. ultimum* var. *ultimum*) in the seedling assay. However, Red Hawk had significantly higher emergence than Zorro when challenged with oomycete species from *Pythium* Clade F ($P < 0.0001$), which is a globally distributed clade containing many important plant pathogens (Lévesque and De Cock 2004). These findings indicate that Zorro may be more resistant to species from the *P. ultimum* complex, whereas Red Hawk may be more resistant to *P. spinosum* (the only “seed rot” pathogen in *Pythium* Clade F). Across temperatures in the seed assay, fewer *Pythium* species caused significantly higher DSI relative to the NIC for Red Hawk than for Zorro.

Though behavior of oomycete isolates was similar across assays, the two assays were fundamentally different, measuring dissimilar parameters under different light, humidity, and nutrient conditions. As a result, the effect of dry bean variety on *Pythium* virulence varied between assays. The differences in seed size, rates of imbibition and germination, assay duration, nutrition of growing substrate, exposure

to light, and availability of moisture may have affected disease development of *Pythium* species on the two dry bean varieties. Results from this study did not show a clear effect of dry bean germplasm across assays on resistance to *Pythium*-induced seedling disease and root rot. However, results do suggest that *Pythium* resistance

Fig. 4. Seed assay mean disease severity index (DSI) of oomycete species on Zorro and Red Hawk dry beans, by temperature. Bars indicate standard errors. Points above the dashed line are significantly different from the noninoculated control (NIC) (Tukey's HSD, $\alpha = 0.05$). Host-oomycete interactions that resulted in significantly different DSI (Tukey's HSD, $\alpha = 0.05$) between temperatures are marked with arrows at the x-axis.

Table 4. Significance of intraspecific variation in virulence, measured by seedling assay root area, length, and dry weight and seed assay disease severity index, for select oomycete species^a

	Seedling assay			Seed assay
	Root area ^b (% of NIC)	Root length (% of NIC)	Root weight (% of NIC)	Disease severity (DSI)
<i>Py. aff. diclinum</i> (3) ^c	41.6–96.0***	44.1–87.4***	48.1–84.2***	0.07–0.12†
<i>Py. aphanidermatum</i> (3)	0.1–41.7***	0.1–39.2***	0.3–51.2***	0.83–0.92**
<i>Py. CAL_2011f</i> (3)	25.2–72.2***	35.1–70.5***	38.5–65.9***	0.10–0.58***
<i>Py. conidiophorum</i> (3)	65.4–110.0***	68.0–91.7***	64.2–97.9***	0.07–0.30***
<i>Py. paroeccandrum</i> (3)	48.6–82.3***	63.2–93.6***	61.9–82.3**	0.14–0.38***
<i>Py. spinosum</i> (4)	2.3–74.0***	2.1–87.9***	23.0–87.4***	0.10–0.80***
<i>Py. sylvaticum</i> (8)	15.5–114.4***	18.2–86.2***	39.5–104.3***	0.07–0.56***
<i>Py. ultimum</i> var. <i>ultimum</i> (3)	4.0–19.3*	5.9–26.3***	14.8–40.7***	0.65–0.89***

^a All oomycete species with significant interspecific variation in root area, length, and dry weight are presented.

^b For each parameter, the lowest and highest means of isolates within the species are expressed. Numbers in parentheses refer to the number of isolates representing each species.

^c Significance of species effect is designated as follows: * < 0.05 , ** < 0.01 , *** < 0.0001 , † = nonsignificant.

in bean varieties may not prove equally effective across all pathogenic oomycete species.

In the seed assay, the DSI of 14 oomycete species were significantly different between 20°C and 26°C on at least one dry bean variety. DSI varied significantly due to temperature, even for oomycete species with DSI that were not significantly different from the NIC. Similar to the current study, previous studies have demonstrated that *Pythium* species have variable optimal temperatures for growth and virulence (Cantrell and Dowler 1971; Kobriger and Hagedorn 1984; Matthiesen et al. 2016). *P. CAL_2011f*, *P. conidiophorum*, *P. irregulare*, *P. spinosum*, and *P. sylvaticum* caused significantly higher DSI at 20°C than 26°C for at least one dry bean variety. Matthiesen et al. (2016) demonstrated that *P. torulosum* caused serious disease on soybean at 13°C, but caused substantially less disease above 18°C, which may explain the absence of symptoms from *P. torulosum* in the current study. Conversely, DSI of five oomycete species was significantly higher at 26°C than 20°C for at least one bean variety, including *Phytophthora cucurbitacearum*, *P. aff. diclinum*, *P. deliense*, *P. ultimum* var. *sporangiferum*, and *P. aphanidermatum*, which previously has been reported to favor temperatures at or above 20°C (Gold and Stanghellini 1985; Thomson et al. 1971; Wei et al. 2010). Assay temperatures may impact whether or not a *Pythium* species is observed to cause disease. Some oomycete isolates in this study may prove to be pathogenic at different temperatures. Though previous studies have described the effect of temperature on *Pythium* virulence as a uniform trait of isolates within each *Pythium* species (Abad et al. 1994; Gold and Stanghellini 1985), a variable effect of temperature on virulence within some species, such as *P. paroeandrum* and *P. ultimum*, was observed in the current study. These findings indicate that different isolates within a species may have different optimal temperatures for disease development. The intraspecific variability in disease responses to temperature could be better understood by evaluating the spectrum of virulence that can be caused by many isolates of a few “seed rot” oomycete species at different temperatures.

Characterizing dry bean-oomycete interactions of representative isolates from *Pythium* species has multiple benefits for production of dry bean. The most virulent *Pythium* species from different symptom groups or clades can be used for evaluation of *Pythium* resistance across new varieties. Determining the most virulent *Pythium* species in dry bean from among *Pythium* species abundant in agricultural fields can also help the development of disease management practices specific to the most harmful pathogens. *Pythium* species that are abundant and highly virulent on dry bean can also be preferentially monitored for changes in fungicide sensitivity. Determining the effects of temperature on *Pythium* species virulence may help growers to improve disease management by adjusting cultural practices that impact soil temperature around the seed, such as planting date (Naseri and Mousavi 2013), planting depth (O'Brien et al. 1991), or tillage practices.

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