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Research Paper

Characterization of *Phytophthora capsici* isolates from lima bean grown in Delaware, United States of America

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Summary. Pod rot of lima bean (*Phaseolus lunatus* L.), caused by the broad host range oomycete *Phytophthora capsici,* is an emerging threat to lima bean production in the mid-Atlantic region of the United States of America (USA). There is little known about survival and spread of this pathogen in the State of Delaware, an area of major lima bean production. Irrigation water was sampled in 2014 and 2015 for the presence of *P. capsici* using baiting methods. Over three seasons, isolations from water sources, weeds, and soil samples did not yield *P. capsici*. However, field samples from symptomatic lima bean, watermelon, muskmelon, pepper, pickling cucumber, and pumpkin yielded 64 *P. capsici* isolates. Characterization of the isolates showed that 42 were of the A2 mating type, 31 were sensitive to mefenoxam, 18 were intermediately sensitive, and four were insensitive to this fungicide. All isolates were pathogenic on the eight lima bean and two snap bean cultivars tested. Three EST-SSR markers, PCSSR19, PCN3, and PCN7, used in combinations of PCSSR19/PCN3 or PCSSR19/PCN7 were significantly associated with mefenoxam sensitivity. This study is the first of its kind in Delaware, providing key information as a basis for effective management of *P. capsici*, including mating type, mefenoxam insensitivity, host range, and survival.

Keywords. Fungicide resistance, oomycete, plant pathogen, mefenoxam.

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INTRODUCTION

The causal agent of pod rot of lima bean, *Phytophthora capsici* (Leonian 1922), infects members of at least 27 plant families, including vegetable crops in the Cucurbitaceae, Solanaceae, and Fabaceae, as well as conifers, weeds,

and tropical crops around the world (Erwin and Ribeiro, 1996; Davidson *et al.* 2002; Gevens *et al*., 2008; Roberts *et al*., 2008; Quesada-Ocampo *et al*., 2009; Granke *et al*., 2012). This heterothallic, hemi-biotrophic oomycete, belonging to the Peronosporales and Pythiaceae, causes root, stem, fruit and crown rot, foliar blight, and stunting on various hosts (Gevens *et al*., 2008; Quesada-Ocampo *et al*., 2016). The pathogen has a broad host range and can cause 50% crop losses in agro-ecosystems (reviewed in Sanogo and Ji, 2012). Lima bean (*Phaseolus lunatus* L.) and snap bean (*Phaseolus vulgaris* L.), however, are the only reported legumes affected by *P. capsici* (Davidson *et al*., 2002; Tian and Babadoost, 2003; Gevens *et al*., 2004). Lima bean is the cornerstone of the Delaware vegetable processing industry and a greater area is grown in the mid-Atlantic region (MAR) than elsewhere in the United States of America (USA). Approximately 5,600 ha of lima bean crops are planted in Delaware annually (https: //www.nass.usda.gov/Statistics_by_State/Delaware/index.php). Lima bean fields are often planted after an early season vegetable crop, such as peas or cucumbers. Planting susceptible vegetable crops in rotation or as a double crop ahead of lima bean in fields with previous histories of *P. capsici* may increase the risk of lima bean pod rot (Hausbeck and Lamour, 2004). Along with lima bean, snap bean crops are also grown in and around Delaware, often as warm season vegetable crops for autumn harvest. In 2017, 890 ha of snap bean crops were grown in Delaware (https: //www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=DELAWARE). Though there are reports of *P. capsici* affecting snap beans in other states of the USA (Gevens *et al*., 2008; McGrath and Dillard, 2011), occurrence of snap bean pod rot in Delaware has not yet been reported.

The asexual sporangia of *P. capsici* produce motile zoospores that may spread in irrigation water or rain (Ristaino *et al*., 1992; Granke *et al*., 2012). Zoospores may remain viable for hours or days in water (Roberts *et al*., 2005). Dispersal of *P. capsici* sporangia by wind is not frequent and the dispersal to other fields solely by wind is unlikely (Granke *et al*., 2009). Sexual oospores are produced in the presence of A1 and A2 mating types (MT), and oospores survive in soil for variable times (Babadoost and Pavon, 2013). oospores may survive in infested soil and plant debris for more than 5 years, resulting in infective propagules after crop rotations with non-host crops (Lamour and Hausbeck, 2001). This could result in significant genetic variation of outcrossing populations in the field (Lamour *et al*., 2012). Weeds may be alternative hosts in the absence of host crops, and host weeds found and reported in the eastern USA for *P. capsici* include black nightshade (*Solanum americanum*, *S. nigrum*), common purslane (*Portulaca oleracea)*, velvet leaf (*Abutilon theophrasti)*, and Carolina geranium (*Geranium carolinianum)* (Tian and Babadoost, 2003).

Studies on *Phytophthora capsici* have demonstrated broad genetic diversity, demonstrated with genetic fingerprinting and molecular markers (Hu *et al*., 2013; Lamour *et al*., 2012), with genetic clustering (Granke *et al*., 2012), and with physiological race testing in pepper in New Mexico (Glosier *et al*., 2008; Monroy-Barbosa and Bosland, 2011). These results indicate that physiological races exist in *P. capsici*. A physiological race is defined as "a subdivision of a pathogen species, particularly fungi, distinguished from other members of the species by specialization for pathogenicity in different host cultivars" (Kirk *et al*., 2001). Cultivar differentials are generally used to identify physiological races.

Management strategies for *P. capsici* include applications of the fungicide mefenoxam, which has been used widely for *Pythium* and *Phytophthora*. However, prolonged use of this compound has contributed to the emergence of fungicide insensitivity in *P. capsici* populations and some *Pythium* species (Brent and Hollomond 1998; Parra and Ristaino 1998; Weiland *et al*., 2014). Fungicide insensitivity in *P. capsici* to mefenoxam, hymexazol, cyazofamid, pyrimorph, and flumorph has been reported (Jackson *et al*., 2012; Pang *et al*., 2013, 2016; Jones *et al*., 2014). In the Mid-Atlantic region of the USA, mefenoxam insensitive isolates of *P. capsici* from lima bean were first reported in 2008 (Davey *et al*., 2008). Traditionally, mefenoxam sensitivity of an isolate is tested using *in vitro* assays with fungicide-amended media (Parra and Ristaino, 2001; Hausbeck and Lamour, 2004; Keinath, 2007). While these assays are still performed with *P. capsici* isolates (Qi *et al*., 2012; Ma *et al*., 2018), we wished to develop a molecular marker-based method to rapidly identify mefenoxam sensitivity.

In the last two decades, molecular markers have been used to identify specific traits or changing populations in *Phytophthora* species. Lamour and Hausbeck (2001) used amplified fragment length polymorphism (AFLP) markers to resolve population dynamics of a recombinant field population of *P. capsici*. Additionally, Pei-Qing *et al*., (2013) identified four expressed sequence tag, simple sequence repeat (EST-SSR) markers to unravel diversity in *P. capsici* populations in China. While Hu *et al*., (2014) reported a sequence characterized amplified region (SCAR) marker that can distinguish mefenoxam insensitive populations and sensitive populations of *P. nicotianae*, to date there are no reported markers capable of distinguishing between insensitive and sensitive *P. capsici* isolates.

Surface water sources used for irrigation in Michigan, Georgia, New York, and other states in the USA have been shown to carry *P. capsici* (Bush *et al*., 2003; Roberts *et al*., 2005; Wang *et al*., 2009; Gevens *et al*., 2007; Jones *et al*., 2014). Though the pathogen is not known to overwinter in irrigation water sources, water may aid in its spread. The objectives of the present study were to characterize *P. capsici* isolates collected from irrigation water sources, weed and crop hosts and soil samples from the states of Delaware and Maryland (USA) to (1) determine *P. capsici* mating types, mefenoxam insensitivity, host range, and dispersal; (2) develop molecular markers to distinguish mefenoxam sensitive isolates from insensitive isolates; and (3) detect the presence of other *Phytophthora* species. This information would aid development of effective disease management strategies. We undertook these studies in order to better understand this economically important, broad host range pathogen on an important crop in our region; if lima bean cannot be produced profitably in the MAR, other processing vegetables such as peas, snap beans, sweet corn and spinach, would not be produced, resulting in severe economic losses to the region (Evans *et al*., 2007).

MATERIALS AND METHODS

Sampling: baiting, infected field samples, water, weeds, and soil samples

Thirteen surface water sources including lakes, streams, and naturally-fed ponds were sampled in Kent and Sussex counties in Delaware during the summers of 2014, and 2015. Soil proximal to water sources, known potential *P. capsici* weed hosts, and infected fruits from grower fields were also sampled in the summers of 2014, 2015 and 2016. Baiting traps were each constructed by attaching a polyethylene foam cylinder (5.7 cm in diameter) to a mesh laundry bag (30 cm × 30 cm) with a zipper. Two unripe pears, one whole eggplant, and two cucumbers were placed in each trap as bait along with two rhododendron leaves. Fruits and leaves were surface-sterilized using 0.825% sodium hypochlorite (NaOCl) with two ≅10 µL drops of Tween 20 (Agdia Inc.) for 100 mL of the solution, for 2 min, and washed with sterile distilled water before adding to the bait bag. Bait bags were kept in the water for 4–6 d.

Numerous infected plant samples from 18 crop fields were collected or obtained from the University of Delaware Plant Diagnostic Clinic, from locations in Delaware and Maryland over the 3 year study. Extensive field sampling was carried out in one location in Bridgeville, Delaware (field 17, Table S1) in the summer of 2016. This field was planted with pickling cucumber (*Cucumis sativus*) early in the growing season, then double-cropped with lima beans later in the same growing season. At least 20-25 samples from different locations of this field were sampled to avoid sampling bias that could lead to non-recovery of isolates belonging to both mating types.

Twenty-six water samples (two 1 L bottles per source) were collected from 13 water sources. Two rhododendron leaves were added to one water bottle (1 L) and incubated in the dark. After 3 d, water was removed, and the leaves were washed with sterile distilled water and kept under moist conditions at 25°C for 3 d until lesions developed. The remaining water was vacuum filtered in 100 mL batches using 3.5 cm diam. (P5 Fisher Brand, 3 µm) filter papers. Filter papers were then placed face down on PARP-V8 selective medium (Ferguson and Jeffers, 1999) and incubated at 25°C in the dark for 3–5 d for colony development.

Two 15 g soil samples were collected near the edge of each surface water source. For each 15 g of soil, 200 mL of distilled water was added, mixed to uniformity, and then filtered through several layers of cheesecloth to remove soil and other debris. Filtered water samples were then processed using the vacuum filtration technique described above.

Roots of weeds near water banks were collected if the plants were previously reported as hosts for *P. capsici*. Carolina geranium (*Geranium carolinianum*) and common purslane (*Portulaca oleracea*) were the only weeds known to be hosts for *P. capsici* that were present near the water sources sampled. Roots were washed thoroughly with running tap water and surface sterilized as described previously.

All samples collected (except the water and soil samples) were rinsed in sterile distilled water and dried under ambient laboratory conditions in a biosafety hood. Tissues from margins of lesions from each of these samples were placed on PARP-V8 medium and incubated at 25°C in the dark for 3–5 d.

Morphological identification and generation of single zoospore cultures

Hyphal tips of potential *P. capsici* isolates growing on PARP-V8 medium were transferred to 60% strength potato dextrose agar (PDA; BD Difco) and unclarified V8 medium (600 mL distilled water, 163 mL unfiltered V8 juice, 12 g agar, 1.7 g $CaCO_3$) to examine isolate growth patterns. Sporangium formation was induced in 7–10 d-old cultures grown on unclarified V8 medium using Chen-Zentmeyer salt solution (Chen and Zent-

meyer, 1970), and the isolates were identified based on morphometric characteristics (Erwin and Ribeiro 1996; Gallegly and Hong 2008). Single zoospore cultures (SZC) were obtained from field isolates positively identified as *P. capsici* (morphologically and molecularly). Sporangium formation was induced on 10 d-old *P. capsici* isolates with Chen-Zentmeyer salt solution. After 18–20 h, the solution was decanted and 10 mL of sterile distilled water added. Plates were maintained at 4°C for 1 h followed by 15–30 min at room temperature, to induce release of zoospores. Twenty to 50 µL of each zoospore suspension was spread on a water agar plate, incubated at 25°C for 2–3 d, and then examined under a dissecting microscope. Three germinating zoospores per each hyphal-tipped field isolate were each transferred to a low strength PDA plate, and then to a V8 agar plate. Single zoospore cultures were stored long term in sterile distilled water within sterile screw-capped tubes each containing two hemp seeds and three cucumber seeds, and were maintained at 20°C.

DNA extraction

For each isolate tested, a 7 mm diam. plug of actively growing 7-d-old culture was added to 25 mL of lima bean broth (Calvert *et al.*, 1960) and incubated at 25°C for 3 d on an orbital shaker. Mycelial mats growing on broth were harvested using vacuum filtration through Whatman #1 filter paper. After removing the original colonized agar plugs, the mycelial mats were washed with sterile distilled water, lyophilized, and then kept in -80°C until processed. DNA was extracted with a Wizard Genomic DNA purification kit (Promega). DNA was dissolved in 100 µL of DNA rehydration solution and diluted to a final concentration of 25 ng μL^{-1}.

Molecular identification and primers

DNA samples were amplified using two sets of *P. capsici* specific primers (Zhang *et al.*, 2006, Lan *et al.*, 2013) to confirm identification. PCR reactions were carried out with a nested PCR protocol with universal ITS1/ITS4 for the first PCR round, and the primers PC-1 (5'-GTCTTGTACCCTATCATGGCG-3') and PC-2 (5'-CGCCACAGCAGGAAAAGCATT-3') for the second PCR round, as described by Zhang *et al.* (2006). The expected amplified product size was 560 bp. Isolates were also characterized using the *P. capsici* specific primers Pc1F (5'-GTATAGCAGAGGTTTAGTGAA-3') and Pc1R (5'-ACTGAAGTTCTGCGTGCGTT-3'), as described in Lan *et al.* (2013). The expected product size was 364 bp. A 25 µL PCR reaction mixture containing 2 µL of template DNA (25 ng μL^{-1}), 5 µL 5× LongAmp *Taq* Master Mix (New England Biolabs), 300 µM dNTPs, 0.4 µM primer, and 2.5 U LongAmp *Taq* DNA Polymerase (New England Biolabs) was used for marker amplification. The PCR amplification protocol used was as follows: initial denaturation at 94 °C for 10 min, 35 cycles of 94°C for 30 s, 56–60 °C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. PCR products were separated on a 2% agarose gel containing ethidium bromide in 10× Tris-Borate-EDTA buffer. Electrophoresis was carried out at a constant 58 V for 2 h and DNA bands were visualized under a UV trans-illuminator. A 100 bp DNA ladder (New England Biolabs) was used to determine amplicon sizes. DNA of isolates confirmed as *P. capsici* were then tested with one Inter-Simple Sequence Repeats (ISSR) marker and five SSR markers, selected from published *P. capsici* population markers, to identify polymorphic molecular markers using a subset of five isolates (Wang *et al.*, 2009, Pei-Qing *et al.*, 2013). One polymorphic marker reported in Pei-Qing *et al.* (2013), PCSSR19 (F-5'-GTCTTCGCTAAAGCCTCCG- 3', R- 5'-AGATGGCCAACAGCGGTTA-3'), showed co-segregation with mefenoxam sensitivity in our population, so this was utilized for further study. Polymorphism of a marker was initially tested with five isolates with varying mefenoxam sensitivity (two mefenoxam sensitive and three mefenoxam insensitive isolates). Markers showing possible co-segregation with mefenoxam sensitivity were further tested with a small subset of 18 isolates before screening all 64 isolates. PCSSR19 primers were searched using BLAST in Fungidb (http: // fungidb.org/fungidb/), and matched one predicated gene in *P. capsici* (PHYCA_548602T0), which when searched using BLAST in NCBI, was shown to be most similar (84% similarity at nucleotide level) to an RNA helicase from *P. infestans*. This coding sequence was then utilized to design additional primers pairs (designated PCN3 and PCN7), using the Primer3 program (Koressaar *et al.*, 2007; Untergasser *et al.*, 2012). The three primer pair sets PCSSR19, PCN3, and PCN7 were then tested on a small set of 18 isolates, and the amplification products were found to be polymorphic between sensitive and insensitive isolates. These three primer sets, PCN3 (F-5'-CGTGGCTTAACCAGTGTTCT-3', R-5'- GACGGTCATAACCACCGTAG-3'), PCN7 (F-5'-CGTTTTCCTACCGATTCCAA-3', R-5'-GACGCGGTACGTATGCAGAT-3'), and PCSSR19, were therefore used to screen all isolates. Conditions for PCR reactions were as follows: initial denaturation at 94°C for 10 min, 35 cycles of 94°C for 30 s, 56–60°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. Products

of PCR were separated on a 4% agarose gel (low range ultra agarose; Biorad) containing ethidium bromide in 10× Tris-Borate-EDTA buffer (Fisher Scientific). Electrophoresis was conducted at a constant 58 V for 5 h, and DNA bands were visualized under a UV trans-illuminator. Isolates were screened with markers two additional times to confirm the correct banding pattern.

Mating type determination

Mating type of each *P. capsici* isolate was determined by pairing single-zoospore cultures with known isolates of *P. capsici*, [A1 (10193) or A2 (SP98) MT], as described by Gevens *et al.* (2007). A 7 mm plug of 7-d-old SZC of *P. capsici*, grown on a V-8 plate at 25°C, was placed 3 cm from a 7 mm plug of either the A1 or A2 MT tester isolate on a V8 plate, and incubated at 25°C in the dark. Plates were examined under a compound microscope for the presence of oospores after 14 to 21 d.

Mefenoxam sensitivity

Mefenoxam sensitivity was determined as outlined in Gevens *et al.* (2007). A 7 mm plug from an actively growing plate of SZC of *P. capsici* was placed on three, 100 mm diam. plates of V8 agar amended with 100 ppm of mefenoxam (Technical grade, or Ridomil Gold SL, 45.3%, Syngenta) and two V8 non-amended controls. The active ingredient in Ridomil Gold SL mefenoxam at 45.3%, with the remainder of the formulation being proprietary additives. Technical grade mefenoxam was dissolved in acetone and Ridomil Gold SL was dissolved in sterile distilled water prior to adding to the media cooled to the touch (approx. 55°C). Three SZC from each of the 64 *P. capsici* field isolates were tested with Ridomil Gold SL. After incubating plates at 23°C in the dark for 3 d, perpendicular colony diameters were measured, and percent average growth of an isolate was obtained by comparing growth on V8 plates with growth on V8 amended with mefenoxam. Isolates were rated as follows: sensitive isolates had <30% growth compared to the controls, intermediately insensitive isolates had 30-90% growth compared to the controls, and insensitive isolates had >90% growth compared to the controls, as described in Gevens *et al.* (2007). The experiment was repeated. All three of the SZC's from each isolate were observed and recorded for mefenoxam phenotype, because in several instances, one SZC differed from the other two. To account for these discrepancies, homogeneity of variance among SZC's from the two experiments were assessed with Bartlett tests. Statistical significance of the mefenoxam reactions between SZC was also tested using a Student's t test at $\alpha = 0.05$, using JMP Pro 13 (JMP®, Version *13*. SAS Institute Inc., 1989-2019). The correlations between mefenoxam sensitivity and MT were tested with the Pearson coefficient at $\alpha = 0.05$ also using JMP Pro 13.

Pathogenicity of Phytophthora capsici *to lima bean and snap bean*

Plants of the commercial lima bean cultivars 'Cypress', 'C- Elite Select', 'Fordhook (FH) 242', 'Bridgeton', 'Eastland', 'Maffei (M) 15', '8-78', and '184-85' were each inoculated with isolates of *P. capsici* collected during 2014-2016. A lima bean plant introduction PI477041 from Arizona, determined to have some resistance to *P. capsici* in field and greenhouse inoculations (N. Gregory, personal communication) was also included. Plants were grown under greenhouse conditions at 25°C with a photoperiod of 10 h and light intensity of 2500-3500 $\mu Em^{-2} sec^{-1}$ until pod set. Accession PI477041 was grown with a photoperiod of 12 h to induce flowers and pods. Young, flat pods were collected and challenged, as described by McGrath (2009) for snap bean pods, with slight modifications. Pods were surface sterilized using a 0.825% NaOCl solution (as above) and completely dried under a laminar flow hood. Two pods were placed on each Petri plate (100 × 15 mm Fisherbrand™) lined with a filter paper moistened with sterile distilled water. A 7 mm diam. plug of an actively growing *P. capsici* culture, grown on V8 plates for 7 d at 25°C, was placed on each pod. Plugs of V8 culture medium without the pathogen were placed on pods as experimental controls. Plates were sealed with Parafilm and incubated at 25°C and a 10 h photoperiod. Pods were assessed as susceptible or resistant 3 to 5 d post inoculation. *Phytophthora capsici* was re-isolated from symptomatic tissue placed on PARP-V8 selective media. The experiment was repeated twice. Two isolates identified as a *Pythium*/*Phytopythium*-like spp., isolated from water baits, were also tested on the differential group of lima bean cultivars, using the method described above, to determine whether they were pathogenic on lima bean.

Snap bean cultivars 'Provider' and 'Caprice' were inoculated with isolates of *P. capsici* under laboratory and greenhouse conditions. Thirteen *P. capsici* isolates out of the 64 total were tested on young snap bean pods in the laboratory, using the protocol adopted from McGrath (2009). Six pods were tested per isolate and the experiment was replicated once. Greenhouse testing was done for three of the 13 isolates used in the laboratory testing to observe *in planta* reactions. Lima bean culti-

vars 'M15' and '8-78' were used as positive controls, and greenhouse assays consisted of four plants of each cultivar per isolate. Isolates were grown on V8 agar plates for 14 d, which were subsequently flooded with sterile distilled water, with sporangia being dislodged with a glass rod. Pods were spray-inoculated with a spore suspensions (3×10^4 mL^{-1}) until run off and kept under moist conditions (plastic chamber on a greenhouse bench at 24°C with misting for 1 min every hour during daylight hours from 7 am to 7 pm) until symptom development. Control plants were sprayed with sterile distilled water. Symptoms were evaluated 4 an 12 d post-inoculation. Pods from a total of four replicated plants were examined, and rated as susceptible if they showed typical symptoms of *P. capsici* infections. This experiment was repeated once. Infections were confirmed by re-isolating *P. capsici* from inoculated and symptomatic tissues.

RESULTS

Isolate collection and identification

More than 200 microbial isolates were collected from irrigation water, soil samples, plant hosts, and weed hosts. However, none of the isolates recovered from direct sampling of irrigation water, baited fruits, leaf baits, soil samples, or roots of weed hosts were *P. capsici*. *Pythium*/*Phytopythium*-like isolates were mainly recovered from surface water sources in late August and early September. *Phytophthora capsici* isolates were recovered only from the infected plant material from crop fields. The numbers of *P. capsici* isolates collected from infected plant material from these fields were 22 in 2014, 16 in 2015, and 26 in 2016, for a total of 64 isolates. All the isolates were positively identified morphologically as *P. capsici*, and yielded the expected 560 and 364 bp bands, when amplified, respectively, with *P. capsici* specific primers PC1/PC2 (used in the nested PCR with ITS1/ITS4 primers) and Pc1F/Pc1R primers. Isolate information is presented in Table 1.

Mating type (MT) determination testing

Isolates belonging to both MT of *P. capsici* were recovered in all three years. Of the 64 *P. capsici* isolates recovered, 42 out of 63 field isolates were A2 MT, with 17 A2 MT isolates recovered from lima bean (Tables 1 and S1). Mating types A1 and A2 were recovered from four field locations in Delaware (fields 8, 12, 17, and 18), including the intensively sampled field in Bridgeville Delaware in the summer of 2016 (Table S1; field 17, 20 samples).

Pathogenicity on commercial lima bean and snap bean cultivars

Pathogenicity testing of *P. capsici* isolates showed all 64 isolates were virulent on all the commercial lima bean cultivars tested ('Cypress', 'C- Elite Select', 'Ford-

Table 1. Numbers of *Phytophthora capsici* isolates, their host sources, mating types, mefenoxam sensitivities, and locations, for isolates collected in the 2014, 2015 and 2016 field seasons.

Host	Number of isolates	Mating types		Mefenoxam sensitivity[a]					Location	
		A1	A2	S[b]	S-IS[c]	IS[d]	IS-I[e]	I[f]	DE[g]	MD[h]
Lima bean	27	10	17	12	2	9	2	2	20	7
Pumpkin	5	2	3	3	1	1	0	0	4	1
Pepper	4	0	4	2	1	1	0	0	3	1
Muskmelon	1	1	0	0	0	0	0	1	1	0
Watermelon	15	2	13	7	2	5	0	1	15	0
Pickling cucumber	12	7	5	7	3	2	0	0	12	0
Totals	64	22	42	31	9	18	2	4	55	9

[a] Mefenoxam sensitivity of field isolates are categorized based on the reaction of the three single zoospore cultures tested for each field isolate.
[b] Sensitive.
[c] Sensitive to Intermediately sensitive.
[d] Intermediately sensitive.
[e] Intermediately sensitive to Insensitive.
[f] Insensitive.
[g] Delaware.
[h] Maryland.

hook (FH) 242', 'Bridgeton', 'Eastland', 'Maffei (M) 15', '8-78', and '184-85'), evidenced by clear signs of white sporulation. Isolate PC67 is an accurate representation of susceptible interactions on these cultivars (Figure. 1). Controls showed no signs of infection or discolouration (Figure 1). Most isolates were pathogenic on PI477041, with the exceptions of isolate PC33 (no sporulation), PC61 and PC62 (reduced sporulation and browning), and PC51 and PC37 (reduced sporulation) (Figure S1). Two of the *Pythium/Phytopythium*-like isolates identified in this study were tested on pods of the lima bean cultivars, and were non-pathogenic, with the exception of isolate Phy4 on 'Eastland' (Figure S2). The two snap bean cultivars, 'Caprice' and 'Provider', were susceptible to the 13 isolates tested in the laboratory, and to a subset of three representative isolates tested in the greenhouse, showing similar signs of sporulation. Control pods inoculated with sterile distilled water showed no symptoms. *Phytophthora capsici* was re-isolated from the infected pods.

Mefenoxam sensitivity

Since the replicates were homogeneous between experiments (Bartlett test - F ratio = 0.0046, $P = 0.9954$), the results from both experiments were averaged to obtain the mefenoxam reaction for the isolates. Of the 64 field isolates tested, 31 were sensitive (S), 18 intermediately sensitive (IS), and four insensitive (I) to Ridomil Gold SL (Table 1). Single zoospore cultures derived from eleven isolates showed variability in their reaction to mefenoxam (Tables 1 and S1). Nine isolates showed S-IS phenotype (of these, six were statistically significant at $\alpha = 0.05$) while two isolates showed IS-I phenotype (neither were statistically significant at $\alpha = 0.05$) (Table S1). There was no significant correlation between the mefenoxam sensitivity and the mating type at $\alpha = 0.05$ (Pearson coefficient = -0.23 $P = 0.063$, Table S1). We wished to determine whether SZCs showed different responses to Ridomil Gold SL (45.3% mefenoxam) versus technical grade mefenoxam (97% ai). This assay showed that SZCs from 14 field isolates exhibited the same reactions while SZCs from several other field isolates gave variable reactions to both mefenoxam formulations (Table S2). For example, all three SZCs derived from two field isolates (PC33 and PC43) gave different reactions, while two out of three SZCs from one field isolate (PC34), and one of three SZCs from four field isolates (PC36, 44, 46, and 55) showed different reactions to both formulations (Table S2). However, only the reactions observed for SZCs of PC33 and PC34 were statistically significant at $\alpha = 0.05$, suggesting that reaction of SZCs

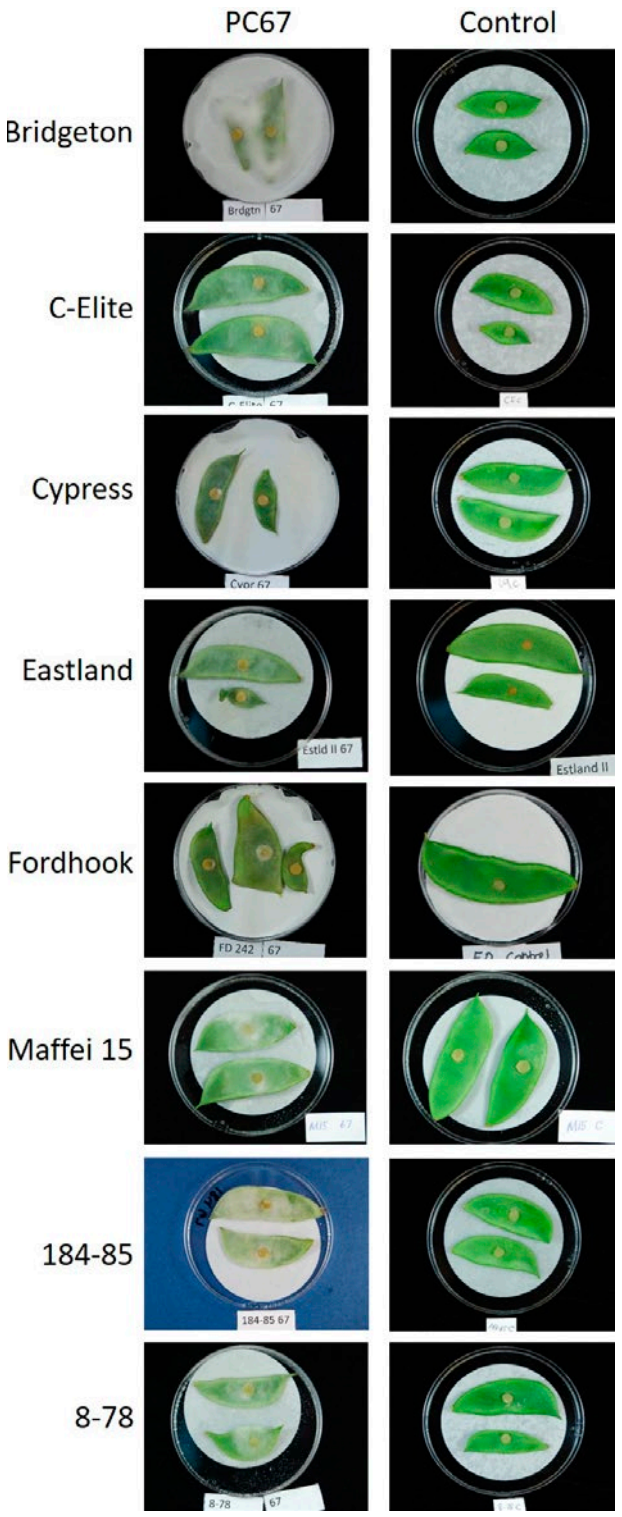

Figure 1. Lima bean pod assay. Pathogenicity testing of *Phytophthora capsici* isolate 67 on lima bean pods from eight different cultivars. Isolate 67 inoculations resulted in sporulation on all eight cultivars, representative of all of other isolate reactions with the lima bean cultivars. Pods were photographed 5 d post inoculation. The experiment was repeated once, with similar results.

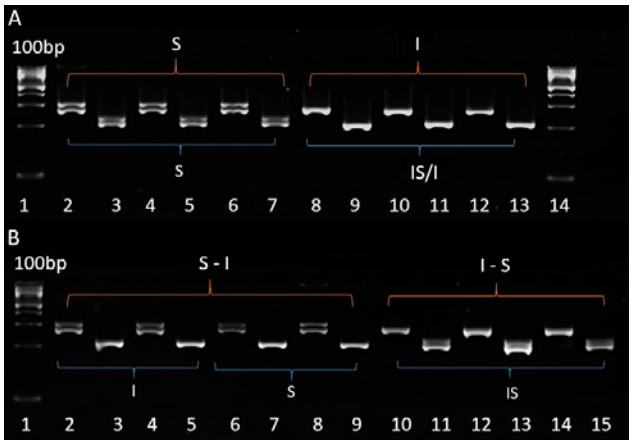

Figure 2. Segregation of mefenoxam sensitive and intermediately sensitive/insensitive *Phytophthora capsici* isolates, based on the SSR markers PCSSR19 and PCN3. S = sensitive, IS = intermediately sensitive, I = insensitive. Predictions based on the markers are shown above the fragments, and mefenoxam sensitivities of the isolates are shown below the fragments. Fragment sizes for each primer pair are as follows: PCSSR19 (S, 263 bp, 281 bp and 325 bp; IS/I, 252 bp and 270 bp) , PCN3 (S, 240-250 bp and 205-215 bp; IS/I, 205-215 bp). (A) Isolates showing the same segregation pattern for both PCSSR19 and PCN3. Lanes 1 and 14 are the 100 bp ladder. Even numbered lanes 2 to 12 are marker PCSSR19, odd numbered lanes 3 to 13 are PCN3. (B) Isolates exhibiting different segregation patterns with the markers PCSSR19 and PCN3. Lane 1, 100 bp ladder, even numbered lanes 2 to 14, PCSSR19, odd numbered lanes 3 to 15, PCN3.

to the technical grade mefenoxam and to Ridomil Gold SL were very similar.

Three EST-SSR markers tested, PCSSR19, PCN3, and PCN7 (which co-segregates with PCN3), were significantly correlated with mefenoxam sensitivity at $\alpha = 0.05$ (Pearson coefficients 0.507 (P = <0.001) for PCSSR 19 and 0.402 (P = 0.001) , for PCSSR 19 and for PCN3 and PCN7). Mefenoxam sensitive isolates yielded three bands of 263, 281, and 325 bp, while the isolates assessed as IS or I to mefenoxam yielded two bands of 252 and 270 bp with PCSSR19. Mefenoxam sensitive isolates produced two bands with PCN3 and PCN7 (240-250 and 205-215 bp), while mefenoxam insensitive isolates showed only the 205-215 bp band with PCN3 and PCN7. Mefenoxam sensitivity was predicted more accurately when both markers yielded the same banding pattern (Figure 2A, Table S3). Accuracy of sensitivity prediction when both markers predicted sensitivity was 96%, or for insensitivity was 85%. Twenty-five out of the 26 field isolates predicted to be mefenoxam sensitive by both markers were sensitive to Ridomil Gold SL in plate-based assays, and 11 out of the 13 field isolates predicted to be insensitive to mefenoxam exhibited insensitivity to Ridomil Gold SL. When PCSSR19 predicts insensitivity and PCN3 predicts sensitivity to mefenoxam, the plate-based reaction was IS (Figure 2B, Table S3). Isolates IS to mefenoxam could at times yield the bands for sensitivity with the PCN3 marker. Prediction of the actual sensitivity reaction was less accurate when PCSSR19 predicted sensitivity and PCN3 predicted insensitivity (Figure 2B). Based on these there was a 56% probability for the actual reaction to be IS (Table S3). Correlations between the marker combinations PCSSR19/PCN3 or PCSSR19/PCN7 with mefenoxam reaction, not considering instances where PCSSR19 predicted sensitivity and PCN3 predicted insensitivity, were significantly greater (Person coefficient 0.745 (P < 0.001).

DISCUSSION

Phytophthora capsici is an emerging threat to lima bean production in the main USA growing regions of Delaware, Maryland and Virginia. The goal of the present study was to isolate, identify and characterize many lima bean-infecting pathogen isolates in Delaware, and to determine their sensitivity to the widely-applied fungicide mefenoxam. In the course of 3 years, we recovered 64 *P. capsici* field isolates out of 200 samples collected. To identify a potential source of this important pathogen in grower fields, samples were collected from irrigation water sources, weed hosts, soils, and infected plant material. Of all these sources, *P. capsici* isolates were only obtained from infected plant material in crop fields. We were unable to identify any *P. capsici* isolates from irrigation sources, although they have been identified from irrigation sources in Florida, Michigan, and Georgia (Roberts *et al.*, 2005; Gevens *et al.*, 2007; Wang *et al.*, 2009). Our baiting method was similar to those of Wang *et al.*, (2009), although they used infected bait fruits to then inoculate pepper stems, which resulted in increased numbers of *P. capsici* isolates recovered. They observed that plating directly from bait fruits can favour organisms which outgrow *P. capsici*. Thirty seven isolates representative of the isolates recovered from irrigation sources were assessed for molecular identification. Details of this identification are listed in Table S4. None of these species were known pathogens of lima bean, though some where known to cause diseases on other hosts. Many of the isolates recovered from irrigation sources were morphologically similar to *P. capsici*. Based on the sequence data for the maternally inherited Cox1 gene and the ITS, however, several of the isolates recovered from water sources in Delaware were identified as the putative maternal parents of *Phytophthora* x *stag-*

num (Yang *et al*., 2014; Table S4). The hybrid species *P.* x *stagnum* was recently described (Yang *et al*., 2014). The paternal parent of *P.* x *stagnum* is reported as *P. chlamydospora*, previously known as *P.* taxon Pgchlamydo, (Hansen *et al*., 2015), and the maternal parent is thought to be an unknown species close to *P. mississippiae*. The present study is the first report of the identification of the putative maternal parent of *P.* x *stagnum* (C. Hong, personal communication). Of the other species recovered from the water sources, isolates of *P. irrigata* have been reported from water sources in other studies (Kong *et al*., 2003; Hong *et al*., 2008). *Phytopythium* is a novel genus in the Pythiaceae, exhibiting morphological characteristics in between *Pythium* and *Phytophthora* (de Cock *et al*., 2015). However, both isolates of the *Pythium*/*Phytopythium*-like species tested on the lima bean cultivars in the present study did not cause disease on pods except for isolate 4 on 'Eastland'. This indicates that these isolates were non-pathogenic on lima bean, but further greenhouse testing is needed, in particular, further testing on the 'Eastland' cultivar.

The presence of both MTs of *P. capsici* in the same field has been reported previously in New Jersey and North Carolina (Papavizas *et al*., 1981; Ristaino, 1990; Dunn *et al*., 2010). Mating type A2 appears to be dominant in Delaware, as approx. 66% of the field isolates tested were of this MT. Our results also demonstrate that both MTs can be present in the same field. This has significant ramifications for management of *P. capsici*, as new strains could quickly emerge due to sexual reproduction. Oospores can be viable in fields for more than 5 years, even after rotations with non-host crops (Lamour and Hausbeck, 2001). Hence, rotations of lima bean with non-host crops could become a less effective management strategy in grower fields in Delaware, if both MTs were present in the same field. *Phytophthora capsici* isolates belonging to both MTs were recovered from a field in Bridgeville, Delaware (field 17), which was planted with pickling cucumber early in the 2016 field season and then with lima bean later in the same growing season. Field 12 also tested positive for *P. capsici* in both 2015 and 2016, with both MTs found in 2016 (Table S1). This indicates that the pathogen survived over time, which could have been due to the presence of compatible MTs leading to formation of oospores are able to survive adverse conditions. Only one isolate was collected in 2015 from this field, and this could possibly be the reason for not detecting both MTs in this field in 2015.

Sampling a field multiple times could affect the accuracy of a study. While large numbers of samples could increase chances of detecting isolates of both MTs and different mefenoxam sensitivities within a field, these could also result in the possibility of sampling clones of individual isolates multiple times. This could affect the ratios for MTs and the accuracy of markers used to predict mefenoxam sensitivity. We sampled fields in multiple locations to offset this issue. Assuming the isolates of one MT and the same mefenoxam sensitivity to be clones of a single isolate within a field (disregarding the year collected and the source), we found 34 isolates of which 23 (68%) were of A2 MT. Though slightly greater, this proportion was is not significantly different from the 66% reported for the 64 isolates ($\chi^2_{df=1} = 0.184$, $P = 0.668$). At the same time, despite having the same MT and mefenoxam sensitivity, isolates collected from different years or different sources from the same field could also be different. Therefore, further analyses using sequence data are required to clearly identify the clones of individual isolates.

Out of 64 isolates collected, 31 were sensitive to mefenoxam, four were insensitive, and 18 were of intermediate sensitivity to the compound. There was variability among the SZCs of the remaining field isolates, indicating that each field isolate was a population of individuals. For example, SZCs of 11 field isolates had S to IS phenotypes, and two SZCs had IS to I phenotypes. Six out of these 11 reactions were significantly different between the individual zoospore isolates. These results indicate that *P. capsici* isolates insensitive to mefenoxam have increased in field populations since 2008, when mefenoxam insensitive isolates were first reported from MAR (Davey *et al*., 2008). This emphasizes the need for alternative fungicides for management of *P. capsici* diseases. We tested a subset of 21 field isolates for reaction to technical grade mefenoxam to determine whether this gave the same result as the commercial formulation Ridomil Gold SL, which contains only 45.3% mefenoxam. Enough technical grade fungicide was available to test three SZCs each for 21 field isolates with both forms of mefenoxam. Results indicated that 91% of the isolates give the same, or very similar, reaction (reactions not statistically different), regardless of whether they were exposed on technical grade mefenoxam or Ridomil Gold SL (Table S2). The two field isolates (PC33 and PC34) that gave significantly different phenotypes ($\alpha = 0.05$) could have resulted from effects of proprietary additives in the commercial Ridomil Gold SL formulation. However, this also suggests that each *P. capsici* field isolate is likely to be a population of individuals; each SZC cultured from the original field isolate, is not necessarily a clone. This assay provided the same phenotypes in two separate replicates. Further experiments should include at least 25 SZC from a single field isolate in plate-based and molecular-based mefenoxam sensitivity assays, to

provide insights into the nature of each field isolate.

Molecular markers were identified as associated with mefenoxam sensitivity, which could be important tools for initial screening of isolates. Mefenoxam sensitivity prediction was more accurate when both markers, PCSSR19 and PCN3, predicted the same reaction to mefenoxam. In a rare case, the two markers were inconsistent with each other, when PCSSR19 predicted sensitivity and PCN3 predicted insensitivity. In these instances, a plate-based assay should be used to confirm the reactions. Isolates PC34 and PC36, which showed S-IS reaction to Ridomil Gold SL, were S to technical grade mefenoxam (Table S2). Both PCSSR19 and PCN3 predicted sensitivity to mefenoxam for these two isolates. These markers are potentially robust tools for rapidly identifying fungicide-insensitive isolates. Future studies will include testing on a larger sample of *P. capsici* isolates than reported here, as well as generation and testing of a segregating population of the pathogen.

Results of the pod assays indicated that all commercial lima bean cultivars used in this study were susceptible to all *P. capsici* isolates tested, including isolates recovered from cucurbits and crops other than lima bean. Susceptibility of all commercial lima bean cultivars highlights one of the biggest disease management challenges. Accession PI477041 exhibited limited sporulation to some *P. capsici* isolates. It is hoped that this line and other resistant landraces can be used in breeding for resistance to *P. capsici* in the future. The detached pod assay could be used as an initial screening system for identifying resistance to this pathogen in future breeding efforts. Extensive greenhouse and field-testing could then be carried out on selected germplasm lines, with reduced cost and resources. Snap bean inoculations in this study indicated that *P. capsici* isolates from Delaware can cause disease on snap beans under optimal conditions for infection. Snap beans are widely grown in Delaware with no reports of *P. capsici* infections in field sites. However, there are reports of *P. capsici* infecting snap bean in other states (Gevens *et al.*, 2008; McGrath and Dillard, 2011). Examining the host range of *P. capsici* in Delaware is important information for farmers, as well as for developing effective management strategies to control *P. capsici* in the MAR.

As a pathogen, *P. capsici* has a broad host range and can attack different host tissues (Hausbeck and Lamours 2004). The presence in a field of both MTs of this pathogen, and isolates which are insensitive to mefenoxam, could result in oospore production and long-term survival of the pathogen, and also in reduced fungicide efficacy against the diseases it causes, posing severe management implications for lima bean production.

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