Plant Disease • 2019 • 103:2751-2758 • https://doi.org/10.1094/PDIS-02-19-0381-RE

Development and Application of Real-Time and Conventional SSR-PCR Assays for Rapid and Sensitive Detection of *Didymella pisi* Associated with Ascochyta Blight of Dry Pea

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Abstract

Didymella pisi is the primary causal pathogen of Ascochyta blight (AB) of dry pea in Montana. Diagnosis of AB is challenging because there are six different species that cause AB worldwide and that can co-occur. Additionally, agar plate identification of *D. pisi* is challenging due to its slow growth rate. Currently, there are no PCR-based assays developed for specific detection of *D. pisi* or any fungal pathogen in the AB complex of dry pea. In this study, we evaluated simple sequence repeat (SSR) primer pairs for their specificity and sensitivity in real-time and conventional SSR-PCR both in vitro and in planta. The specificity of the assay was determined by testing DNA of 10 dry pea varieties, fungal species in the AB complex, and fungal species associated with dry pea. To avoid false-negative results, plant and fungal DNA markers were included as controls in a conventional multiplex SSR-PCR, to amplify any plant or fungal DNA in the absence of the *D. pisi* SSR target. SYBR Green SSR-quantitative PCR (qPCR) detection was conducted using the same primer pairs but in a uniplex format. *D. pisi* was specifically amplified, whereas other fungi and host DNA were not. Also, sensitivity experiments showed that the detection limit was 0.01 ng of DNA of *D. pisi* for both assays and 100 conidia in SSR-qPCR. These assays are valuable diagnostic tools for the detection of *D. pisi.*

Keywords: field crops, fungi, pathogen detection, techniques

In 2017, in total, 1 million acres of dry pea (*Pisum sativum*) were harvested in the United States, representing a farm-gate value of $170 million (USDA-NASS 2017). In Montana during the 2017 cropping season, 190,200 ha of dry pea were harvested, representing a farm-gate value of $42 million. Also, Montana accounted for 47% of the total U.S. dry pea production in 2018 (USDA-NASS 2018). Changes in pathogen populations and the risk of moving pathogens to new areas pose a great risk to the production of high-quality and disease-free dry pea seed required in the export markets.

Six fungal species have been associated with Ascochyta blight (AB) of dry pea (Aveskamp et al. 2010; Barilli et al. 2016; Davidson et al. 2009; Li et al. 2011; Tran et al. 2014; Trivoli and Banniza 2007). All of these pathogens can exist together on a host or independently of each other (Davidson et al. 2009; Trivoli and Banniza 2007). However, *Didymella pisi, Peyronellaea pinodes*, and *P. pinodella* are the only three fungal pathogens associated with AB of dry pea in the Great Plains of North America. In Australia, *Phoma koolunga*, *P. glomerata*, and *P. herbarum* are the additional pathogens associated with the disease. (Aveskamp et al. 2010; Davidson et al. 2009; Li et al. 2011; Tran et al. 2014). These pathogens are associated with shrinking and discoloration of dry pea seed, and lesions on foliar parts of pea plants (Bretag and Ramsey 2001; Bretag et al. 2006; Gossen et al. 2011; Skoglund et al. 2011). In Montana, the predominant fungal species associated with AB of dry pea is *D. pisi* (Owati et al. 2017). *D. pisi* is seed and residue borne and the dry pea plants are susceptible to the pathogen at every growth stage (Davidson et al. 2009, 2011; Gossen et al. 2011). Under conditions favorable to the disease, the pathogen can cause 15 to 30% yield loss in dry pea (Ahmed et al. 2015; Wallen 1965; Xue et al. 1996, 1997).

Current identification methods for *D. pisi* are laborious and time consuming. Identification using an artificial medium such as potato dextrose agar requires between 9 and 12 days for the fungus to grow and sporulate (International Seed Testing Association 2017]; Owati et al. 2017). Furthermore, molecular identification of this pathogen is challenging because of the high sequence similarities of fingerprinting genes such as *internal transcribed spacer* (*ITS*) genes and intergenic sequences that exist among AB-associated pathogens. To date, the use of multiple gene sequences has been used to identify this pathogen (Liu et al. 2016). Confounding diagnostics further is the fact that the other two pathogens (*Peyronellaea pinodes* and *P. pinodella*) associated with AB of dry pea in the Great Plains of North America have symptom expression on pea plants similar to that of *D. pisi.* These challenges complicate diagnosis, reinforcing the need for an accurate and sensitive assay to diagnose this pathogen and track changes in pathogen populations over time.

For both initial and rapid evaluation of AB-diseased plant samples, the detection of *D. pisi* for diagnostic purposes in the field and laboratory have focused on the use of symptoms, the morphology of asexual structures (pycnidia), and sequencing of the fungus ITS region and other conserved genes. The use of artificial media is laborious and time consuming due to the slow-growing nature of *D. pisi* and the need for single-spore isolation to obtain pure cultures (Ahmed et al. 2015; Davidson et al. 2009). Multiloci gene sequencing used for the diagnosis of *D. pisi* is not rapid and requires pure cultures which might take 10 to 14 days for mycelial growth. An additional 3 to 5 days are needed for DNA isolation, PCR, and sequencing of amplicons (Liu et al. 2016). Thus, a rapid, sensitive, and specific pathogen detection method will facilitate pathogen identification and assist with more timely and effective disease management strategies such as tracking changes in pathogen populations and determining the need for targeted fungicide applications (Wang et al. 2006). Early detection of the fungal pathogen on plants will inform targeted fungicide application and, thus, control disease incidence.

Molecular biology techniques, including PCR, have been used widely for detection and identification of phytopathogenic fungal species. Both conventional and real-time PCR assays have been developed for both in vitro and in planta detection and quantification of

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Funding: The study was funded by the Department of Plant Science and Plant Pathology, Montana State University, Regional Pulse Crop Diagnostic Laboratory, Bozeman, MT supported by USDA/Montana Farm Bill 16-8530-1769-CA-FB Pulse Crop Lab.

The author(s) declare no conflict of interest.

Accepted for publication 7 June 2019.

a number of foliar, root, and soil phytopathogenic fungi (Bhat and Browne 2010; Bonants et al. 1997; Cao et al. 2007; Chilvers et al. 2007; Henson et al. 1993; Hughes et al. 1998; Hussain et al. 2005; Keller et al. 1995; Kuzdraliński et al. 2017; Li et al. 2013; Lin et al. 2009; Moricca et al. 1998; Moukhamedov et al. 1994; Niepold and Schöber-Butin 1995; Phan et al. 2002; Wang et al. 2006, 2007; Wang et al. 2015). Zitnick-Anderson et al. (2018) developed a multiplexed quantitative PCR (qPCR) for the identification and quantification of seven *Fusarium* spp. associated with root rot of dry pea. Also, Rojas et al. (2017) developed a qPCR and recombinant polymerase amplification assays for genus- and species-specific detection and quantification of *Phytophthora sojae* and *P. sansomeana* root rot pathogens of soybean from plant tissue and soil samples.

Currently, there is no rapid, sensitive, and specific detection assay developed for in vitro and in planta detection of any of the pathogens in the AB complex of dry pea. Moreover, this hinders diagnosis and epidemiological studies, because several closely related species can cause similar symptoms on dry pea plants. The objectives of this study were to (i) develop a specific, conventional simple sequence repeat (SSR)-PCR assay for the detection of *D. pisi*; (ii) develop an SSR-qPCR assay for specific detection and quantification of *D. pisi*; and (iii) validate the qPCR and PCR assays in the presence of both host materials and other fungal species.

Materials and Methods

Fungal isolates and DNA extraction. Fungal genera associated with AB of dry pea and other genera commonly associated with dry pea and used in this study, including *Didymella*, *Peyronellaea*, *Phoma, Alternaria, Stemphylium, Nigospora, Fusarium, Botrytis, Colletotrichum, Sclerotinia*, and *Cladosporium*, were collected from symptomatic dry pea leaves and seed from Montana, Idaho, and Washington. *Phoma koolunga* DNA was provided courtesy of Dr. Jenny Davidson, South Australia Research and Development Institute, Urrbrae, South Australia, Australia. Single-spore cultures of each isolate were maintained on potato dextrose agar (PDA) at 22°C with a 12-h diurnal photoperiod. Total genomic DNA was extracted from 10-day-old cultures grown on PDA using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, U.S.A.), with modification of the starting process. Fresh fungal mycelium of each isolate (100 mg) from a 10-day-old culture was scraped into a 2-ml screwcap tube (MP Biomedicals, Santa Ana, CA, U.S.A.) containing 450 ml of AP1 buffer. The mycelium was disrupted using the SPEX SamplePrep 2010 Geno/Grinder (SPEX SamplePrep LLC, Metuchen, NJ, U.S.A.) set at 1,500 rpm for 60 s and centrifuged at 13,000 × *g* for 1 min. About 400 ml of lysate was then transferred to a 1.5-ml collection tube. From this stage onward, the protocol followed the manufacturer's instructions. The quality and concentration of extracted DNA were estimated using the NanoDrop 2000c Spectrophotometer at 260 nm (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The DNA samples were stored at −20°C until further use.

Development of conventional SSR-PCR for specific and sensitive detection of *D. pisi*. *Screening of SSR primers for* D. pisi *specificity.* Nine SSR primer pairs (A311, A313, A315, A318, A321, A324, A325, A326, and A342) (Owati et al. 2019) which amplified a single band in 205 *D. pisi* isolates from Montana were selected for this study. These primers were screened for their specificity to *D. pisi* using host plant DNA and other fungal DNA templates in uniplex PCR. Total genomic DNA was extracted from the foliage of 10 widely planted dry pea varieties in Montana courtesy of Dr. Kevin McPhee of Montana State University, Bozeman, using a cetyltrimethylammonium bromide procedure (Abarshi et al. 2010) (Table 1).

The PCR was optimized in a total volume of 25 µl containing 12.5 µl of Dream Taq Green PCR 2× Master mix (Thermo Fisher Scientific), 10 pM each forward and reverse primer, and 2.5 µl of DNA (50 ng). Amplification parameters were 4 min at 94°C, followed by 30 cycles of 30 s at 94°C and 30 s at 56.6°C, and a final extension at 72°C for 5 min in a thermocycler (Bio-Rad iCycler; Bio-Rad Laboratories, Hercules, CA, U.S.A.). The PCR products were analyzed on SYBR Safe-stained 2.5% (wt/vol) agarose gels run in a 1× sodium-borate buffer (Brody and Kern 2004) and exposed to blue light to visualize DNA fragments. The amplicon sizes were estimated using a 100-bp DNA ladder (Thermo Fisher Scientific) (Owati et al. 2019).

Conventional multiplex SSR-PCR for D. pisi *detection.* Only SSR primers pairs that did not amplify host DNA were used for further experiments (Table 2). Each of the four *D. pisi*-specific SSR primer pairs (A311-F/R, A313-F/R, A315-F/R, and A318-F/R) together with fungal ITS and *Rubisco L*-gene (RBCL) primer pairs (Table 2) were tested for compatibility in conventional multiplex detection of *D. pisi.* The ITS and RBCL primers were used to amplify fungal and host plant DNA internal control DNA targets, respectively. DNA was extracted from two fungal-infected dry pea leaf samples, pure cultures of four fungal species in the AB complex, and nine other fungal species of dry pea (Table 3) and used at 50 ng for the PCR to test also for the specificity of the multiplex PCR. The PCR mix and cycling conditions were as described above, except 10 pM each of the ITS and RBCL primers was added to amplify the internal controls (Nassuth et al. 2000; White et al. 1990).

Conventional multiplex SSR-PCR assay sensitivity tests. The sensitivity of the SSR-PCR assay was assessed by diluting DNA extracted from *D. pisi* culture. Genomic DNA of *D. pisi* (100 ng/µl) was serially diluted 10-fold from 100 ng/µl to 10 fg/µl. The diluted DNA was used as a template in a multiplex PCR as previously described with ITS primers to amplify the fungal internal control target. Furthermore, to determine the sensitivity of this assay in the presence of host DNA, *D. pisi* DNA was serially diluted 10-fold from 100 ng to 10 fg in 50 ng of DNA from dry pea (cultivar Aragorn). Diluted DNA was used as a template in a multiplex PCR assay with *D. pisi* SSR, ITS, and RBCL primers to specifically amplify *D. pisi*, fungal ITS, and plant RBCL targets, respectively.

SYBR Green real-time SSR-PCR amplification parameters. SYBR Green qPCR amplifications were performed on the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad Laboratories). The annealing temperature of the assay was optimized in a gradient qPCR. The SYBR Green real-time was optimized in a total volume of 20 µl containing 10 µl of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), 10 pM each forward and reverse primer, and 2.5 µl of DNA (50 ng). Amplification parameters were 4 min at 94°C, followed by 30 cycles of 30 s at 94°C and 30 s at 59°C, and a final extension at 72°C for 5 min.

Specificity and sensitivity tests of the SYBR Green quantitative real-time SSR-PCR assay. *Mycelial DNA.* To determine the specificity of the real-time SSR-PCR assay, 50 ng of mycelial genomic DNA each of the target and nontarget fungal species listed in Table 3 was used individually as a template. Primer pairs A311-F/R and A315-F/R specific to *D. pisi* were evaluated in the validation stage. The quantification cycle (Cq) values of the target and nontarget fungal species were estimated in a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad Laboratories).

To determine the efficiency and sensitivity of the assays, a 10-fold *D. pisi* mycelial genomic DNA serial dilution from 100 ng to 10 fg was amplified using primer pair A311-F/R. Real-time qPCR efficiency was calculated with the formula efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$. The slope was calculated from the linear regression between DNA $\log_{10}$ (template DNA concentrations) and Cq values using the Bio-Rad CFX Manager software package (Bio-Rad Laboratories).

A similar experiment was conducted to determine the detection limit of the real-time qPCR assay in the presence of host plant leaf DNA using primer pair A311-F/R. Mycelial genomic DNA from *D. pisi* was serially diluted 10-fold from 100 ng to 1 fg in 50 ng of genomic DNA from pea leaf (cultivar Aragorn). The Cq values were estimated as previously described.

Conidial DNA. In order to quantify the number of spores that could be detected in an assay, a conidial suspension was obtained from three 15-day-old cultures of *D. pisi*. This experiment was conducted twice. To prepare the suspension, the petri plate was flooded with 10 ml of sterile distilled water and conidia were dislodged using a sterile glass rod. The conidial count was estimated

using a hemocytometer viewed with a phase contrast Leica TCS SP5 imaging system (Leica Microsystems Inc., Buffalo Grove, IL, U.S.A.). The spore concentration was adjusted to 10^5 conidia/ml and serially diluted 10-fold to 10 conidia/ml. DNA was extracted from each spore concentration using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions, with modification of the starting process, and tested with the specific qPCR assays. The efficiency and slope of the assay were computed as previously described.

Validation of the SYBR Green real-time PCR assay. To validate these assays, genomic mycelial DNA of 30 isolates of *D. pisi* (Table 4) isolated from AB symptomatic dry pea seed and plants from Montana were spiked with genomic DNA of dry pea at 20 ng of *D. pisi* + 50 ng of host plant DNA per 20-µl PCR. Two replicates of each isolate were used in this experiment. The samples were tested using the PCR assays according to the methods previously described.

Data analyses. Real-time PCR results were collected and analyzed, and qPCR standard curve plots and DNA concentration correlations were plotted, using the Bio-Rad CFX Manager software package (Bio-Rad Laboratories). The results are presented below.

Results

Specificity and sensitivity of conventional PCR assay. Of the nine SSR primer pairs screened with host pea DNA (data not shown), only four primer pairs (A311-F/R, A313-F/R, A315-F/R, and A318-F/R) (Table 2) did not amplify host DNA of 10 dry pea varieties (Table 1; Figs. 1 and 2) and nontarget fungal pathogens associated with dry pea but specifically amplified and detected *D. pisi* isolates (Table 3). In addition, internal control templates were amplified in the multiplex PCR detection of *D. pisi* with RBCL primers (642-bp product) and ITS (550-bp product) primers when the host plant and nontarget fungal were present, indicating that the primers were compatible with SSR primers in the multiplex PCR (Fig. 3). The detection limit of *D. pisi* in the conventional multiplex PCR in both the presence and absence of host DNA was 0.01 ng of *D. pisi* DNA (Fig. 4).

Specificity and sensitivity of the SYBR Green real-time SSR-PCR assay. The SYBR Green PCR assay was specific and sensitive for the detection of *D. pisi* in the presence of the DNA of both the dry pea host and other fungal species. The qPCR detected all *D. pisi*

Table 1. List of dry pea varieties used in the specificity test of the *Didymella pisi* markers

	PCR amplification using	
Dry pea variety	***D. pisi*-specific markers**	***Rubisco L*-gene markers**
Hyline	−	+
Treasure	−	+
CDC Leroy	−	+
Banner	−	+
Aragorn	−	+
Ginny	−	+
Montech 4193	−	+
MSGA-Jet Set	−	+
Early Star	−	+
Nette 2010	−	+

Table 3. Specificity test panel for conventional multiplex polymerase chain reaction assay

	Amplification[a]		
Fungi species, isolate ID	**PCR**	**ITS**	**Origin**
Alternaria alternata, 15	−	+	Montana
A. alternata, 44	−	+	Montana
A. alternata, 49	−	+	Montana
Botrytis cinerea, 2	−	+	Montana
B. cinerea, 3	−	+	Montana
B. cinerea, 4	−	+	Montana
Cladosporium sp., 84	−	+	Montana
Colletotrichum lentis, 63	−	+	Montana
C. lentis, 74	−	+	Montana
C. lentis, 80	−	+	Montana
Didymella pisi, 01-12	+	+	Montana
D. pisi, 01-13	+	+	Montana
D. pisi, 01-14	+	+	Montana
D. pisi, 01-15	+	+	Montana
Fusarium avenaceum, 6	−	+	Montana
F. avenaceum, 13	−	+	Montana
F. avenaceum, 33	−	+	Montana
Nigrospora oryzae, 8	−	+	Montana
N. oryzae, 20	−	+	Montana
N. oryzae, 25	−	+	Montana
Peyronellaea pinodella, 001	−	+	Washington
P. pinodella, 002	−	+	Idaho
P. pinodella, 003	−	+	North Dakota
P. pinodes, AP1	−	+	Idaho
P. pinodes, AP2	−	+	Washington
P. pinodes, AP3	−	+	Montana
Phoma koolunga, T040	−	+	Australia
P. koolunga, FT07013	−	+	Australia
P. koolunga, FT15012	−	+	Australia
Phoma sp., PH1	−	+	Montana
Phoma sp., PH2	−	+	Montana
Phoma sp., PHM	−	+	Montana
Sclerotinia sclerotiorum, 36	−	+	Montana
S. sclerotiorum, 38	−	+	Montana
Stemphylium vesicarium, 12	−	+	Montana
S. vesicarium, 14	−	+	Montana
S. vesicarium, 41	−	+	Montana
No template	−	−	…

[a] *D. pisi*-specific primers were used for the PCR amplification. ITS = internal transcribed spacer.

Table 2. Primer pairs used for specific amplification of *Didymella pisi*, internal transcribed spacer (ITS), and *Rubisco L*-gene (RBCL)

Primers	Primer sequence (5′–3′)	Repeat motifs[a]	Size (bp)	Reference	Purpose[b]
A311-F	AGCAGGCATTACGTTTAACT	(AGC)6	249	Owati et al. 2019	*D. pisi*
A311-R	GGTAAGATGCGAGTACGAAT	(AGC)6	249	Owati et al. 2019	*D. pisi*
A313-F	ATAACAACCAACCTCTGACG	(ACC)6	486	Owati et al. 2019	*D. pisi*
A313-R	GGAGCAATAGGTGATCTTCTC	(ACC)6	486	Owati et al. 2019	*D. pisi*
A315-F	GGTGGACTGAGTTCTGTGTAG	(TTC)7	199	Owati et al. 2019	*D. pisi*
A315-R	TAGCGTGCTCTTGAGGATTA	(TTC)7	199	Owati et al. 2019	*D. pisi*
A318-F	CTAGAATCGTGCTTGTTGC	(TCG)7	406	Owati et al. 2019	*D. pisi*
A318-R	GAGTCTCCCTGTCTTTGTCC	(TCG)7	406	Owati et al. 2019	*D. pisi*
ITS 1	TCCGTAGGTGAACCTGCGG	NA	550	White et al. 1990	Fungus
ITS 4	TCCTCCGCTTATTGATATGC	NA	550	White et al. 1990	Fungus
RBCL-H680	TGGACTTGATTTTACCAAAGATGATG	NA	642	Nassuth et al. 2000	Plant
RBCL-C1321	TGTCCTAAAGTTCCTCCACC	NA	642	Nassuth et al. 2000	Plant

[a] NA = not applicable.
[b] *D. pisi* = *D. pisi* amplification, Fungus = internal control for fungi DNA, and Plant = internal control for plant DNA.

isolates at 50 ng of DNA with a Cq value range of 19.53 to 20.73. Nontarget fungi species were not detected at 30 cycles (Table 3). The correlation coefficients for the standard curve of the DNAs from *D. pisi* pure cultures and conidial suspension were 0.999 and 0.980, respectively (Table 5). The quantification limit for the qPCR was 0.01 ng of mycelia DNA and 100 *D. pisi* conidia.

Validation of the SSR-qPCR assay. To validate the SSR-qPCR assay, DNA of 30 samples of *D. pisi* isolated from AB-contaminated seed lots mixed with 50 ng of DNA of dry pea plants was evaluated. There was consistency in the sensitivity and specificity of the assay when compared with Cq values of pure culture. The mean Cq values of the samples tested ranged from 18.15 to 19.52, with a standard deviation that ranged from 0.01 to 0.31 (Table 4).

Discussion

AB is a threat to dry pea production in the Great Plains of North America, Europe, and Australia (Ahmed et al. 2015; Chilvers et al. 2007; Davidson et al. 2009; Kaiser et al. 2008; Li et al. 2011; Tran et al. 2014). In Montana, *D. pisi* is the most prevalent fungal pathogen in the AB complex (Owati et al. 2017, 2019). Currently, rapid, specific, and sensitive molecular diagnostic assays for this pathogen are lacking. In this study, a rapid, specific, and sensitive qPCR assay was developed to specifically detect and quantify *D. pisi* using SSR primer pairs. In addition, conventional PCR assays were developed for use in laboratories without qPCR capabilities. Both methods specifically detected only *D. pisi*, not closely related fungal pathogens associated with AB complex of dry pea, other fungal pathogens commonly found affecting dry pea, or host dry pea DNA. The assays were sensitive enough to detect 0.01 ng of genomic *D. pisi* DNA of pure culture. In addition, the qPCR assay was sensitive enough to detect 100 conidia of *D. pisi*. This is four orders of magnitude less than the 10^6 conidia/ml routinely used to cause infection in controlled experiments (Ahmed et al. 2015). This shows the robustness of our assay and suggests that it can be used to detect *D. pisi* from infected, non-symptomatic plants.

Various PCR and qPCR assays have been developed for the diagnosis of crop pathogens (Abdullah et al. 2018; Gramaje et al. 2013; Rojas et al. 2017; Wang et al. 2015; Zitnick-Anderson et al. 2018). Most of the qPCR- and PCR-based detection assays for plant pathogens were developed based on the intergenic sequences and ITS sequences of the ribosomal RNA (Bhat and Browne 2010; Rojas et al. 2017; Wang et al. 2015). This approach does not work well to differentiate closely related pathogens that share high sequence homology such as those associated with the AB complex of dry pea (Phan et al. 2002). The approach presented here, of designing the primers to flank loci of SSRs from contigs generated from Illumina next-generation sequencing reads, was instrumental to its high specificity and sensitivity. SSR loci are ubiquitous and offer a unique target in the fungal genome (Canfora et al. 2016; Owati et al. 2019). This characteristic makes SSR markers unique for the development of specific primers for pathogen detection. The specificity and sensitivity of our results are similar to the results of Canfora et al. (2016), who first reported the use of SSR primer-based qPCR for specific detection of fungal pathogens. The study developed an SSR-qPCR assay for the specific detection and quantification of *Beauveria bassiana* and *B. brongniartii* in culture and soil. To our knowledge, this study represents the first study where

Table 4. SYBR Green real-time PCR validation of *Didymella pisi*-specific assay using genomic DNA of different fungal species associated with dry pea and genomic DNA of *D. pisi* isolates from Montana spiked with dry pea DNA samples

Fungal species	Cq values[a]	Standard deviation[b]
Species associated with dry pea		
Alternaria alternata	ND	nil
A. alternata	ND	nil
A. alternata	ND	nil
Botrytis cinerea	ND	nil
B. cinerea	ND	nil
B. cinerea	ND	nil
Cladosporium sp.	ND	nil
Colletotrichum lentis	ND	nil
C. lentis	ND	nil
C. lentis	ND	nil
Didymella pisi	20.73	0.031
D. pisi	20.37	0.021
D. pisi	19.73	0.019
D. pisi	19.50	0.022
Fusarium avenaceum	ND	nil
F. avenaceum	ND	nil
F. avenaceum	ND	nil
Nigrospora oryzae	ND	nil
N. oryzae	ND	nil
N. oryzae	ND	nil
Peyronellaea pinodella	ND	nil
P. pinodella	ND	nil
P. pinodella	ND	nil
P. pinodes	ND	nil
P. pinodes	ND	nil
P. pinodes	ND	nil
Phoma koolunga	ND	nil
P. koolunga	ND	nil
P. koolunga	ND	nil
Phoma sp.	ND	nil
Phoma sp.	ND	nil
Phoma sp.	ND	nil
Sclerotinia sclerotiorum	ND	nil
S. sclerotiorum	ND	nil
Stemphylium vesicarium	ND	nil
No-template control	ND	…
Isolates from Montana		
D. pisi-01	18.31	0.310
D. pisi-02	19.33	0.028
D. pisi-03	19.33	0.064
D. pisi-04	19.48	0.097
D. pisi-05	19.31	0.019
D. pisi-06	19.23	0.032
D. pisi-07	19.44	0.023
D. pisi-08	19.40	0.018
D. pisi-09	19.37	0.020
D. pisi-10	19.52	0.030
D. pisi-11	19.37	0.021
D. pisi-12	19.19	0.014
D. pisi-13	19.28	0.250
D. pisi-14	19.19	0.123
D. pisi-15	18.89	0.057
D. pisi-16	18.93	0.160
D. pisi-17	18.15	0.143
D. pisi-18	19.34	0.013
D. pisi-19	19.36	0.018
D. pisi-20	19.46	0.041
D. pisi-21	19.35	0.095
D. pisi-22	19.47	0.044
D. pisi-23	19.18	0.127
D. pisi-24	19.39	0.031
D. pisi-25	19.36	0.174
D. pisi-26	19.45	0.092
D. pisi-27	19.28	0.101
D. pisi-28	19.04	0.154
D. pisi-29	19.28	0.186
D. pisi-30	19.16	0.027
No-template control	ND	…

(*Continued*)

[a] Cq = quantification cycle and ND = fluorescent signal not detected.
[b] Based on two replicates.

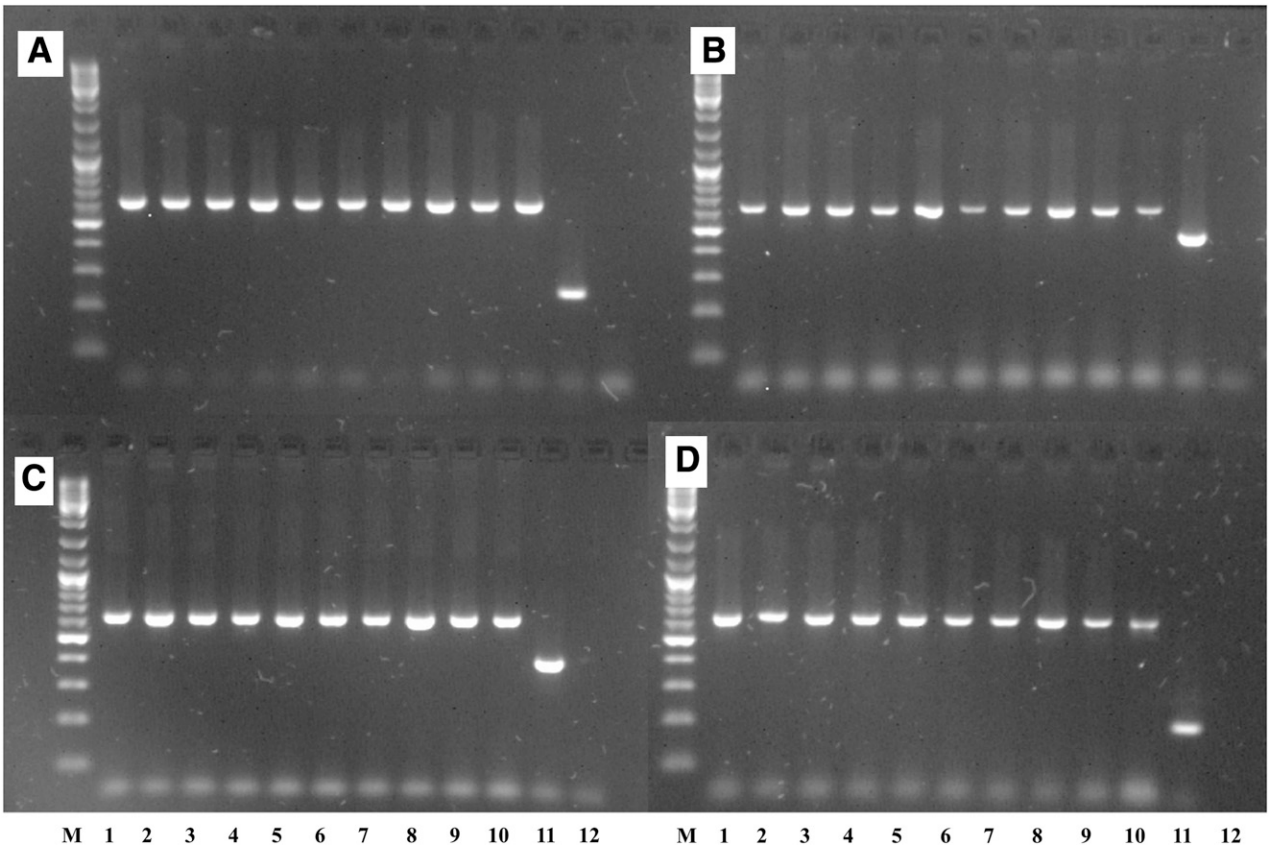

Fig. 1. *Didymella pisi* simple sequence repeat (SSR) primers specifically amplified *D. pisi* but not pea plant DNA in duplex PCR. **A,** Duplex PCR with A311 SSR primer and *Rubisco L*-gene (RBCL) primer pairs. **B,** Duplex PCR with A313 SSR primer and RBCL primer pairs. **C,** Duplex PCR with A318 SSR primer and RBCL primer pairs. **D,** Duplex PCR with A315 SSR primer and RBCL primer pairs. A311, A313, A318, and A315 are SSR primer pairs that specifically amplified 249-, 489-, 406-, and 199-bp products of *D. pisi*, respectively. RBCL primers amplified a 642-bp product from host plant DNA, serving as an internal PCR control. Lane M = 100-bp DNA ladder (100 to 1,500 bp), lanes 1 to 10 = dry pea varieties (Hyline, Treasure, CDC Leroy, Banner, Aragorn, Ginny, Montech 4193, MSGA-Jet Set, Early Star, and Nette 2010, respectively), lane 11 = *D. pisi* DNA positive control, and lane 12 = no template DNA.

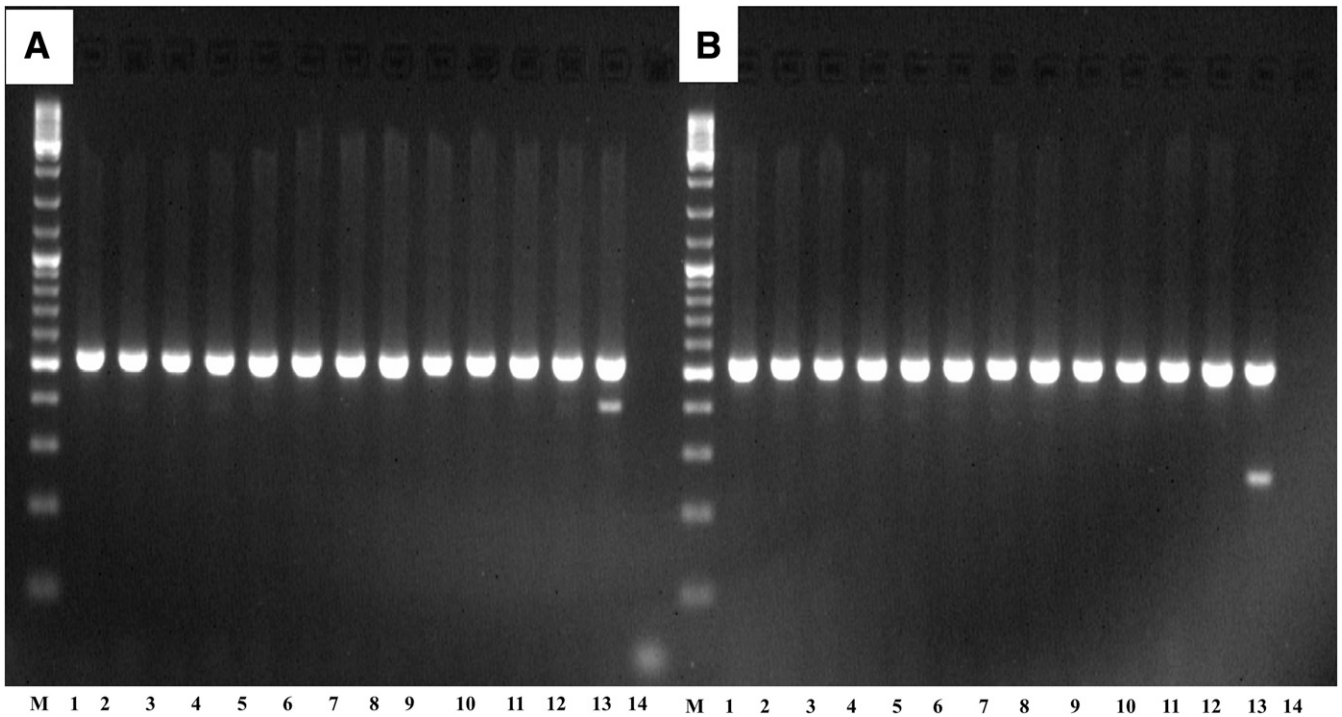

Fig. 2. *Didymella pisi* simple sequence repeat (SSR)-primers specifically amplified *D. pisi* but not any other fungi in duplex PCR. **A,** Duplex PCR with A313 SSR and internal transcribed spacer (ITS)-1 and ITS-4 primer pairs. **B,** Duplex PCR with A311 SSR and ITS-1 and ITS-4 primer pairs. A311 and A313 are SSR primer pairs that specifically amplified 249- and 489-bp products of *D. pisi*, respectively. ITS-1 and ITS-4 generic primers amplified a 550-bp product of all the fungal DNA, serving as an internal fungal PCR control. Lane M = 100-bp DNA ladder (100 to 1,500 bp), lanes 1 to 4 = *P. pinodes* isolates, lanes 5 to 8 = *P. pinodella* isolates, lanes 9 to 12 = *Phoma* sp. isolates, lane 13 = *D. pisi* DNA positive control, and lane 14 = no template DNA.

this novel approach was used to develop specific markers for the detection and quantification of a member of the AB complex in dry pea or AB causal pathogen in another pulse crop.

The need for consistent and accurate PCR-based diagnostic assays for the detection of plant pathogens cannot be overemphasized. The consequences of inaccurate diagnosis can range from the misinformed use of pesticides to hindrances in international trade. Some of the previously developed PCR and qPCR assays for the detection of plant pathogens lacked the inclusion of internal controls (Bonants et al. 1997; Bhat and Browne 2010; Cao et al. 2007; Chilvers et al.

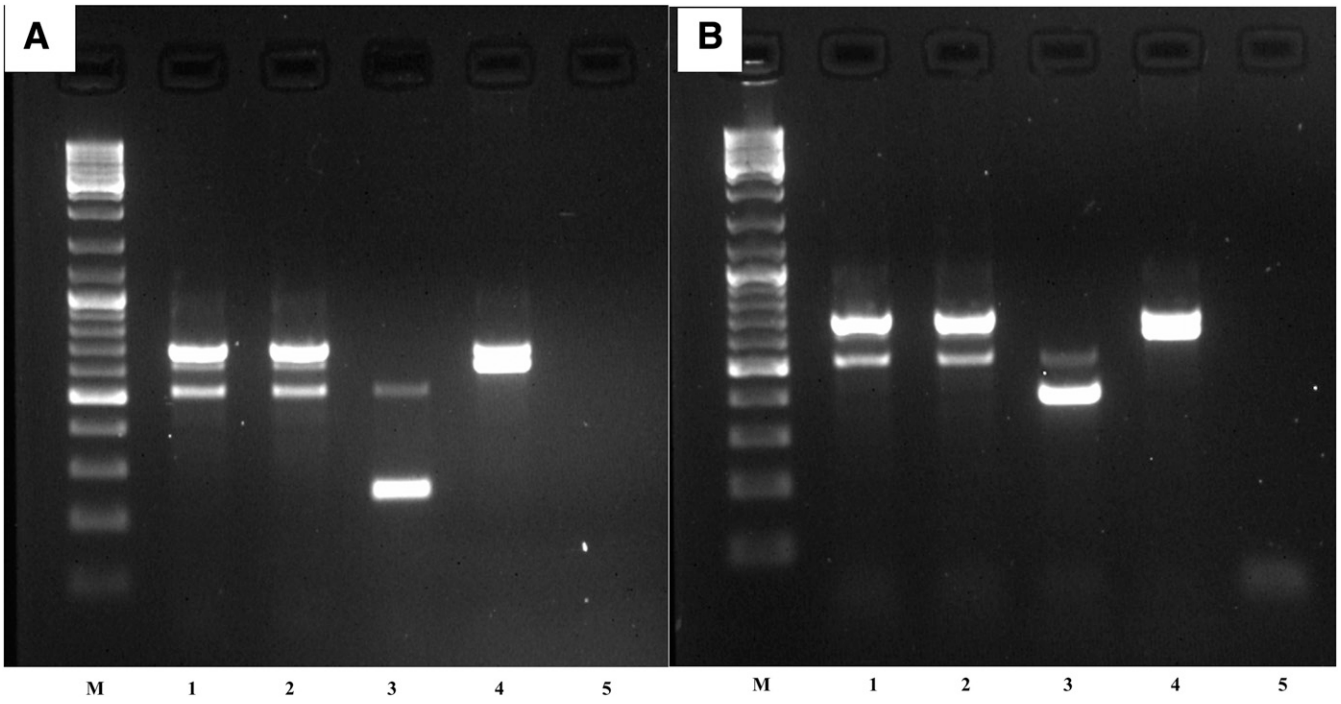

Fig. 3. Multiplex PCR detection of *Didymella pisi*, fungal internal transcribed spacer (ITS), and host plant *Rubisco L*-gene (RBCL). **A,** Multiplex PCR with A311 simple sequence repeat (SSR), ITS-1/ITS-4, and RBCL primer pairs. **B,** Multiplex PCR with A313 SSR, ITS-1/ITS-4, and RBCL primer pairs. Primer pairs A311 and A313 are SSR primer pairs that specifically amplified 249- and 489-bp products of *D. pisi*, respectively. ITS-1 and ITS-4 = generic primers that amplified 550-bp ITS product of any of the fungal DNA, serving as an internal fungal PCR control. Lane M = 100-bp DNA ladder (100 to 1,500 bp), lanes 1 and 2 = dry pea leaf samples infected with *Alternaria alternate*, lane 3 = *D. pisi* DNA positive control, lane 4 = healthy dry pea leaf sample, and lane 5 = no template.

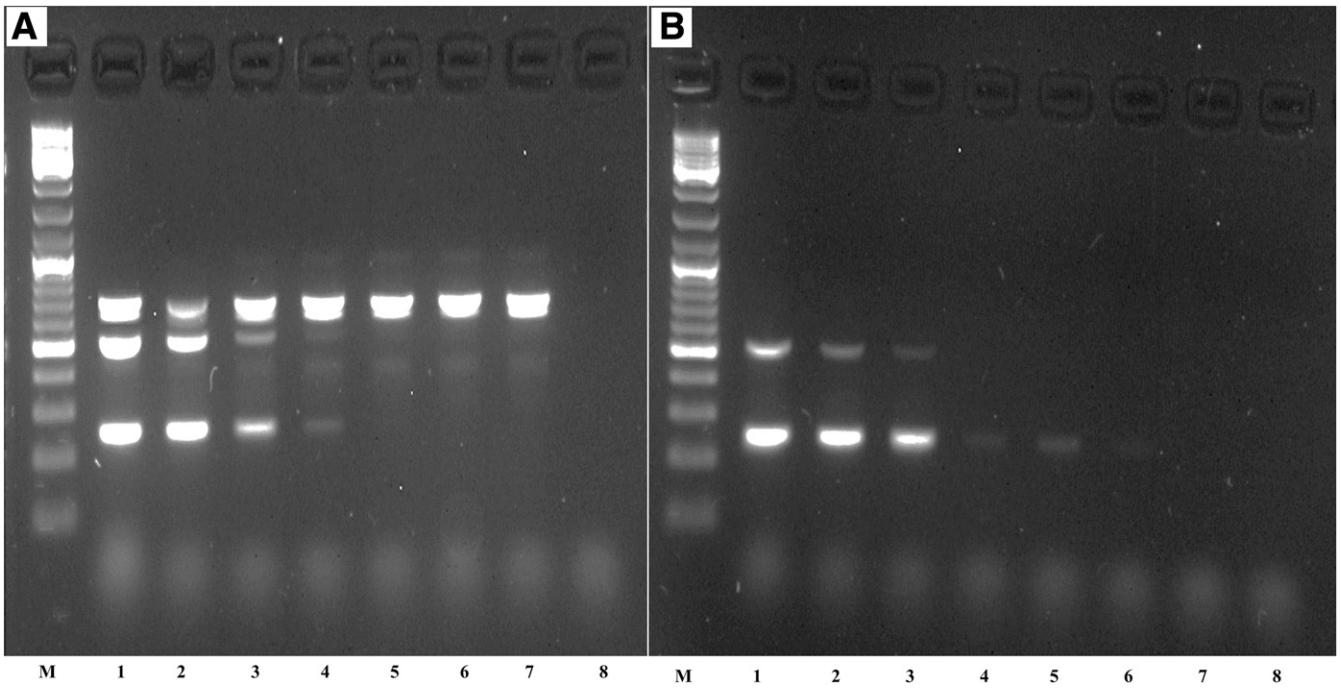

Fig. 4. Sensitivity of multiplex PCR detection of *Didymella pisi* in the presence and absence of host plant DNA. **A,** Multiplex PCR with A311, internal transcribed spacer (ITS)-1/ITS-4, and *Rubisco L*-gene (RBCL) primer pairs. Lane M = 100-bp DNA ladder (100 to 1,500 bp), lanes 1 to 7, 10-fold serial dilution of *D. pisi* DNA from 100 ng to 0.00001 ng in 50 ng of DNA of pea (Aragorn), and lane 8 = no template. **B,** Duplex PCR with A311 and ITS-1 and ITS-4 primer pairs. Lane M = 100-bp DNA ladder (100 to 1,500 bp), lanes 1 to 7, 10-fold serial dilution of *D. pisi* DNA from 100 ng to 0.00001 ng. A311 and A313 are simple sequence repeat primer pairs that specifically amplified 249- and 489-bp products of *D. pisi*, respectively. ITS-1 and ITS-4 = generic primers that amplified 550-bp ITS product of any of the fungal DNA, serving as an internal fungal PCR control.

Table 5. Efficiency of real-time PCR using *Didymella pisi*-specific primer on different sources of DNA

Sources of DNA	Efficiency (%)	Slope	Intercept	R^2	Cq values (min–max)[a]
D. pisi pure mycelial culture	96.5	−3.408	26.280	0.999	19.2–28.5
D. pisi conidial suspension	115.6	−2.996	32.433	0.980	17.5–29.7

[a] Minimum to maximum (min–max) linear range of quantification cycle (Cq) values.

2007; Wang et al. 2006). Internal controls are essential in the assay to control for background amplification and prevent false-negative reports. Hence, our PCR-based assay was designed to multiplex the detection of *D. pisi* with those of ITS and RBCL, which are internal control targets of fungi and plants, respectively. This approach is consistent with other PCR and qPCR assays, where internal controls are included in the assays for the detection of *Fusarium virguliforme*, the causal agent of soybean sudden death syndrome, and *Phytophthora sojae* and *P. sansomeana*, root rot pathogens of soybean (Rojas et al. 2017; Wang et al. 2015).

The development of the assays presented here is timely. Montana shares local and international borders with North Dakota and Canada. The pathogen composition of AB complex of dry pea differs geographically. In North Dakota and Canada (Alberta, Saskatchewan, and Manitoba), the pathogen complex consists of *Peyronellaea pinodes*, *P. pinodella*, and rarely *D. pisi* (Ahmed et al. 2015; Chilvers et al. 2009; Gossen et al. 2011; Sivachandra-Kumar and Banniza 2017). The proximity of these regions and exchange of germplasm is anticipated to influence changes in population dynamics of the pathogen complex (Sivachandra-Kumar and Banniza 2017). The qPCR and PCR assays developed in this study allow for rapid, accurate identification and quantification of *D. pisi*. These tools will be used for epidemiological studies and monitoring changes in pathogen composition. These assays may be used to screen for AB resistance in dry pea, where visual estimation or culture-based methods are difficult or not practical. In addition, the assays can be used to rapidly screen fungal spore traps to understand the distribution of the pathogen and inform the choice of disease management strategy. Furthermore, these assays present an important tool for monitoring changes in the pathogen population. This concern is heightened because the fungal pathogens associated with AB of dry pea in Canada and North Dakota were reported to be more aggressive than *D. pisi* (Kraft et al. 1998). Also, populations of *P. pinodes* are at a risk to develop insensitivity to frequently used fungicides such as quinone outside inhibitor fungicides (Gossen et al. 2014). This type of information will inform improvement on AB management strategies in Montana's dry pea fields. Finally, the *D. pisi*-specific primers could potentially be multiplexed with specific primers for other members of the complex.

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